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# THE ANTI-HISTAMINE ACTIVITY OF THYMOXYETHYLDIETHYLAMINE AND N'ETHYL-N'DIETHYLAMINOETHYLANILINE AS JUDGED BY THE GASTRIC RESPONSE TO HISTAMINE

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The work of Staub (1, 2, 3) indicated that among a series of phenoxyethers studied in regard to their neutralizing effect on certain histamine reactions thymoxyethyldiethylamine and *N*'ethyl-*N*'diethylaminoethylaniline<sup>1</sup>, had superior qualities. It was apparently Staub's opinion that these substances might act mainly by preventing the contraction of smooth muscle and that, while not truly specific, they did have a preferential inhibitory action on histamine contraction of smooth muscle. Rosenthal and his associates (4, 5) have expressed the belief that thymoxyethyldiethylamine is a specific antagonist to histamine in regard to certain of its actions. In addition they reported that this material could produce analgesia in the conscious animal. The latter finding was in accord with Rosenthal's (6) view that histamine might be the peripheral chemical mediator of pain.

To determine whether these substances were truly histamine antagonists or only prevented smooth muscle reactions in a non-specific way, it appeared to us that pertinent information might be found in their effect on the secretory response of Heidenhain pouches to histamine. We have also made a few observations on the toxicity and other reactions of these drugs. To conserve space we shall designate thymoxyethyldiethylamine m.p. 119-123°C. (2-isopropyl, 5-methylphenoxyethyldiethylamine) as *Drug I* and *N*'ethyl-*N*'diethylaminoethylaniline m.p. 125-129°C. (*N*-phenyl-*N*-ethyl, *N*'diethyl, ethylenediamine) as *Drug II*.

**GASTRIC SECRETION.** Healthy dogs possessing functioning Heidenhain pouches and operated on not less than 3 months previously were used. Each experiment was done in duplicate (using two dogs) and repeated at least once. Five sets of observations were made:

1. Study of the gastric response to a single dose of histamine administered subcutaneously (usually 0.2 mgm./kgm.) the animal having received one or other of the drugs in dosage of 25-75 mgm./kgm. subcutaneously, 20 minutes previously.

2. Study of the response of the gastric secretion, maintained relatively constant by histamine administered subcutaneously (0.1 mgm./kgm. every 20 minutes), to the subcutaneous administration of the drug (25 mgm./kgm.).

3. Study of the response of the gastric secretion, maintained by histamine

<sup>1</sup> The samples of thymoxyethyldiethylamine and *N*'ethyl-*N*'diethylaminoethylaniline were obtained through the kindness of the Abbott Laboratories and Dr. S. R. Rosenthal, respectively.

administered subcutaneously (0.1 mgm./kgm. every 20 minutes), following the intravenous injection of the drug (4 mgm./kgm.).

4. Study of the gastric response to histamine administered subcutaneously (0.2 mgm./kgm.), the pouch having been previously filled with 1 or 2% solutions of one or the other drug for 5 or 30 minutes.

5. Study of the gastric response to histamine administered subcutaneously (0.2 mgm./kgm.), the latter having been incubated for 20 hours in a 1% solution of one or the other drug.

The results of 20 experiments on the gastric pouch dogs are summarized quite briefly by stating that neither drug had a demonstrable effect on the secretory

TABLE 1

		NORMAL			DRUG I, SUBCUT., 25 MG.M./KG.M.		
		Volume	Free acid	Total acid	Volume	Free acid	Total acid
		cc. per 20 min.	mill. equiv.	mill. equiv.	cc. per 20 min.	mill. equiv.	mill. equiv.
Dog No. 1	Resting secretion	0.1	0		1.7	126	133
		12.0	132	146	12.0	126	134
	Histamine 0.2 mgm./kgm.	16.5	132	148	16.0	140	146
		6.0	116	144	19.0	144	150
Dog No. 130	Resting secretion	1.3	60	90	0.6	0	50
		18.4	132	140	20*	124	136
	Histamine 0.2 mgm./kgm.	12.1	132	138			
		4.2	130	140			
Dog No. 92	Resting secretion	1.2	52	70		0	
		6.4	138	146	4.6	128	136
	Histamine 0.2 mgm./kgm.	12.4	142	150	12.0	128	136
		6.2	140	148	11.0	142	148

\* 30-minute sample.

response to histamine administered subcutaneously in the quantities indicated. The mass of the experimental data indicated that neither the quantity nor the acidity of the secretion was affected. The results shown in table 1 are representative of the experiments in which one dose of histamine was used. In one experiment in which Drug II was given subcutaneously (26 mgm./kgm.) there was a delay in the secretory response of about one-half hour. This result was never observed again and the animal had shown an early mild toxic reaction to the drug. Drug II was not tested in a higher dosage than 26 mgm./kgm. subcutaneously as in the dog receiving 75 mgm./kgm., convulsions prevented the collecting of the samples.

We have considered the justifiable criticism that a diminution of the gastric

response to histamine is too severe a test for the anti-histamine activity of any drug. This could be said knowing the minute amounts of histamine that may cause the stomach to secrete. We had thought of determining the amounts of histamine necessary to produce a gastric response with and without the previous administration of the drugs, but in our experience the response of the gastric pouch with minimal doses of histamine is extremely variable in itself. The absence of effect on the characteristic secretory response to one dose of histamine, whether the drug was given subcutaneously or in high concentration into the pouch, and likewise absence of effect on a secretory response maintained by multiple doses of histamine, have made us doubt whether these drugs were truly anti-histamine substances. There was no evidence of *in vitro* inactivation of histamine by either of the two drugs, which is in accord with the observations of other investigators.

**TOXIC REACTIONS.** *Intravenous administration.* Doses of 1-2 mgm./kgm. of either drug caused no objective signs in dogs unless the occurrence of defecation and urination, which frequently occurred about one-half hour after administration, was due to the drug. In one animal 5 mgm./kgm. of Drug I caused a momentary collapse followed by an appearance of malaise and anxiety of about two hours duration.

*Subcutaneous administration.* In many of the experiments on the gastric pouch dogs doses of 25 mgm./kgm. were given, usually without apparent reaction. With Drug I one animal at this dosage showed a motor reaction similar to a "running fit," another showed a recurrent nodding *tic* of the head. Defecation occurred in some instances and more rarely vomiting was noted. In one dog 40 mgm./kgm. of Drug I caused no visible disturbance whatever. Four dogs were given 75 mgm./kgm. in 2% solution of Drug I in multiple sites subcutaneously. This produced vomiting, apparent malaise and mild ataxia in all animals; the symptoms were maximal in about  $\frac{1}{2}$  hour and had worn off in about 3 hours. One dog was given 75 mgm./kgm. of Drug II and it developed recurrent severe convulsions, ataxia, hyperirritability and possibly hyperesthesia. Intravenous anaesthesia by a barbital derivative was required to control the convulsions. Twelve hours later the animal was still unable to walk on account of ataxia and apparent weakness, and still showed a hyperirritability to moving objects in its environment. Only by the fourth day was recovery nearly complete.

*Oral administration.* One dog was given a gelatin capsule containing 240 mgm. (20 mgm./kgm.) of Drug I. Fifteen minutes later the animal began to retch, and at short intervals during the next hour vomited a frothy mucus. The animal appeared generally ill but without any definite objective signs. As this reaction was apparently due to gastric irritation and absorption was uncertain, this method of administration was not tried again.

**ANALGESIC EFFECT.** We have found that quantitative differences in cutaneous sensibility are very difficult to assess in the dog. The response to pin prick over the general skin surface of our normal animals has been extremely variable, even from one moment to another. The response to pin prick on the toe pads or

pressure on the feet has fairly constantly produced evidence of pain in the form of whining, withdrawal of the foot, and rapid turning of the head toward the affected area. The pupillary response to pain has not been a particularly useful test in our experience.

Four dogs receiving Drug I in doses of 75 mgm./kgm. subcutaneously were tested for analgesic effect by application of induced currents, by pricking, and by pinching. The threshold for regular reflex flexion on electrical stimulation of the dorsum of the foot was ascertained before injection of the drug at another site. After injection the animals still responded to the same stimulus but with incomplete regularity. Likewise the dogs still responded by leg flexion to pinching of the toes. The responses to pricking over the entire body surface were reduced but not abolished. These effects were seen a half hour after injection and persisted for about 2 hours. Smaller doses, 25-40 mgm./kgm., were without demonstrable effect on pain. In the higher dose levels Drug I appears to have a general analgesic effect, which according to our tests is incomplete.

In one dog, 21 mgm. of Drug I was injected into the left femoral artery and no analgesic effect was seen in this leg. A 1% solution of Drug I placed in the conjunctival sac produced hypesthesia of short duration (15-30 minutes). In this concentration the drug did not produce any evidence of conjunctival irritation.

In regard to Drug II, there was never any evidence that it had any effect on cutaneous sensibility except that in the one dog receiving 75 mgm./kgm. there was the possibility of hyperesthesia.

**REACTIONS IN THE HUMAN SKIN.** Histamine dihydrochloride (0.025 mgm.) injected into the same bleb in the epidermis made 10 minutes previously by either drug (0.1 cc. containing 0.1 mgm.), produced a wheal and flare similar to that of a control reaction to the same amount of histamine in normal skin. Histamine of the same amount injected intradermally in a region where 1 mgm. of either drug had been injected subcutaneously 10 minutes previously also produced a wheal and flare similar to a control. The blebs produced by the injection of either of the drugs intradermally were anaesthetic only over the raised portion of the epidermis. No anaesthesia was produced in the regions where the drugs were given subcutaneously, but in one instance hypesthesia was noted. The small doses used are probably of no consequence in determining the local anaesthetic value of the drugs, but we were not primarily interested in this. The histamine wheals were sensitive to pin prick irrespective of whether they were produced in an area previously injected by drug or in an area of normal skin. In our experience, histamine dihydrochloride intradermally produced sharp pain but only of momentary duration.

**PROTECTIVE ACTION IN GUINEA PIGS.** To give some assurance that the drugs, new in our hands, could produce similar results in different laboratories, the protective action against histamine shock was tested in a small series of guinea pigs (3 groups of 3 animals). The dose of intravenous histamine dihydrochloride used was 1 mgm./kgm.

A subcutaneous dose of 40 mgm./kgm. of Drug I given 20 minutes previous to the histamine gave some protection against the severe dyspnoea and though the animals showed collapse, they recovered completely after 2 hours.

A subcutaneous dose of 40 mgm./kgm. of Drug II given 20 minutes previous to the histamine gave complete protection so that no appearance of shock developed at all. The three controls, given the same intravenous dose of histamine died in a few minutes, showing the characteristic histamine shock picture.

COMMENTS. Absence of inhibitory effect of Drug I upon the gastric secretory response is in confirmation of the report of Loew and Chickering. These workers employed a smaller dose of histamine and found that a somewhat larger histamine response occurred with Drug I than without. They did not study Drug II.

We wish to call attention to the fact that we have not tested preparations of these compounds from sources other than those mentioned and it is not beyond possibility that impurities in material from one or another source in manufacture may have influenced either our own results or those of others. It is necessary to consider also the possibility of deterioration of the drug but this is considered unlikely. It may be of significance in respect to impurities, that the drugs we used did not cause any conjunctival irritation, while the samples used by Staub did. The fact that the material we employed was in our hands able to give protection against acute histamine poisoning in the guinea pig, as found by investigators using other of these substances, argues against any marked difference in the drugs used.

#### SUMMARY

The substances, thymoxyethyldiethylamine and *N*'ethyl-*N*'-diethylaminoethylaniline, have not had demonstrable specific histamine antagonism, as tested by the gastric secretory response of Heidenhain pouch dogs to histamine stimulation. This fact, together with a survey of the reported work, would indicate to us that these materials have a non-specific protective action against smooth muscle contraction. It seems probable that they have a strong preferential inhibition on the histamine type of smooth muscle reaction. With large doses of thymoxyethyldiethylamine administered subcutaneously some evidence of lowered pain sensitivity was observed. No complete analgesia was noted.

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# A COMPARISON OF THE EFFECTS OF EPINEPHRINE ON BLOOD PRESSURE WHEN INJECTED INTO A VEIN AND WHEN INJECTED INTO THE MARROW CAVITIES OF BONE<sup>1</sup>

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An attempt to stimulate the bone marrow of patients suffering from pernicious anemia by injecting an aqueous solution of liver extract into the sternal marrow cavity was made by Josefson (1). He describes the accompanying symptoms as being similar to those noted upon the intravenous injection of the product Tocantins (2) and Tocantins and O'Neill (3) report that blood, glucose and other fluids, when injected into the marrow cavity of the tibia of rabbits and of the sternum of man were almost immediately "absorbed" into the general circulation. By X-ray studies in dead rabbits Tocantins (2) showed that if mercury is injected into the marrow cavity of the tibia it rapidly enters the veins in the bone cortex and the deep femoral vein. He also noted the fact that if either Congo red dye or fluorescein is injected into the tibia it can be recovered within 10 seconds in the plasma of the blood drawn from the right heart.

Potent drugs soluble in water or physiological saline were injected into the marrow cavities of various animals by Macht (4). The subsequent "absorption" of the drug was indicated by the characteristic systemic effects produced. He believed all of the drugs which he studied were "promptly absorbed" through the bone marrow. He also introduced epinephrine in oil into medullary canals, and he observed a marked and long-sustained rise in blood pressure, an effect which could not be achieved by the injection of such an oil solution into skeletal muscle.

Since injections of saline, glucose solution and blood have been made into marrow cavities of bones in human beings and since similar injections of drugs in aqueous as well as in oily suspensions have been made in experimental animals, we believe a thorough knowledge of what happens to these substances when so administered is most important. The injection of oily suspensions into the marrow cavities of bones should be fully studied to determine whether the oil is retained in the bone marrow and subsequently slowly "absorbed" or whether it passes directly into the venous circulation with the possibility of producing oil emboli in other organs.

**METHOD.** Dogs under ether anesthesia and decapitated cats were used to determine if there was a difference in action when equal quantities of epinephrine are injected into a vein or into the bone marrow. The blood pressure was recorded from the right carotid artery with a mercury manometer. Heparin was used as the anticoagulant. While under ether anesthesia the cats were decapitated according to the method described by Sherrington (5). Respiration was maintained by a motor driven respirator.

<sup>1</sup> This research was made possible by a grant from Parke, Davis & Co. for research in science.

Two aqueous solutions of epinephrine hydrochloride were used in each series of experiments. In the experiments on dogs 1:1,000 and 1:10,000 and in those on cats 1:100,000 and 1:1,000,000 dilutions were used. The drug was injected into the left femoral vein and after its effect was recorded and after the blood pressure had returned to the control level for some minutes, the same amount of drug in the same concentration and volume was injected at the same rate into the marrow cavity of the left tibial bone. This routine was later repeated with the other dilution. In these experiments the only variable was the volume of fluid injected. One hundred and eighty-six injections were made in dogs and 110 in cats. These were distributed equally between the intravenous and the intramedullary routes. In each experiment the latent period, the rise in blood pressure and the duration of increased blood pressure were determined. The pointed end of a No. 18 syringe needle with an obturator was forced by pressure and a rotary drilling motion through the shaft of the bone into the marrow cavity of the left tibia about 1.5 to 2.0 cm. below the medial condyle and slightly medial to the crest. When the needle is in the marrow cavity the withdrawal of the obturator usually causes blood to appear in the outlet of the needle. All injections into the marrow cavity were made through this hollow needle. In those experiments in which the pressure within the marrow cavity was studied with a water manometer, there was no noticeable increase in pressure during the injection of the drug.

Rabbits and cats under ether or urethane anesthesia and etherized dogs were used in the experiments in which we attempted to determine whether the material injected into the marrow cavity remained in the bone marrow to be absorbed or whether it passed through the marrow spaces into the venous circulation. The left common and deep femoral veins were exposed and during the experiment they were watched closely for changes in color and for the presence of oil while it was being injected into the bone marrow.

Mineral oil, epinephrine in oil, pitressin in oil and some essential oils in 0.01 to 1 cc. doses were studied. By means of a stop watch the time was determined from the start of the injection to the time of the appearance of the oil as it passed through the deep femoral vein. In some experiments 1 cc. of epinephrine in oil (2 mgm. per cc<sup>2</sup>) was injected intravenously and then into the bone marrow and in other experiments the injections were made in the reverse order. The changes in the blood pressure produced could thus be compared.

**RESULTS.** A comparison of the changes of blood pressure when small volumes of epinephrine hydrochloride are injected into a vein and when they are injected into the bone marrow of a cat is shown in figure 1. It will be noted at 1, 2 and 5, that the intravenous injections of a 1:100,000 dilution of epinephrine (0.001 mgm. per kilogram) caused an average increase in blood pressure of 46 mm. Hg. The same dose injected into the bone marrow at 3 and again at 4 caused an average increase in blood pressure of 28 mm. Hg (61% of the intravenous response).

The average increases in blood pressures when the same amounts of epinephrine are injected into a vein and when injected at the same rate and volume into the marrow cavity of a bone in dogs and cats are shown in table 1. It will be observed that as the volume of fluid injected is decreased the blood pressure response is not greatly altered when the injection is made intravenously but it decreases sharply when injections of a similar nature are made into the bone marrow.

Our experiments by direct observation of the deep femoral vein during injections of mineral oil, epinephrine in oil or pitressin in oil into the bone marrow indicate that these substances enter the venous system too rapidly to get there by a process of "absorption." If 1 cc. of any of the above oily substances is injected

moderately rapidly into the tibial bone in a dog, cat or rabbit the oil will be seen in the common femoral vein within one second after the beginning of the injection. It can be seen flowing in the venous blood stream in the deep femoral vein as long as the administration lasts and it usually disappears upon completion of the injection. It was also noted that if the general circulation was poor more time was needed for the oil to appear in the deep femoral vein. This is due to the fact that the flow of blood carrying the oil is slower and more time is required for the oil to travel from the spaces in the bone marrow to the point in the femoral vein where it is visible.

The injection of 2 mgm. of epinephrine in 1 cc. of peanut oil into the bone marrow of dogs and cats caused an average rise in blood pressure of 24 and 27%

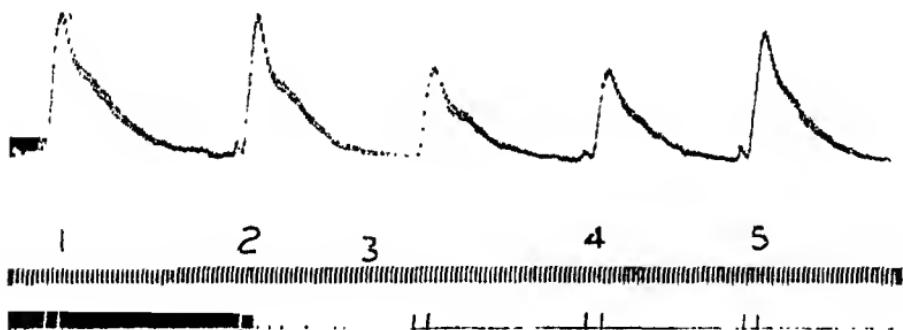


FIG. 1. DECAPITATED CAT, 2.4 KGm.

Upper record is that of blood pressure, below it the time in 6 second intervals and zero blood pressure. The bottom record indicates the interval between the injection and the onset of action. At 1, 2 and 5, epinephrine hydrochloride in a (0.001 mgm. per kilogram) 1:100,000 dilution was given intravenously. At 3 and 4 the same quantity of the same solution was injected into the bone marrow of the tibia.

respectively with an average duration of action of 15.5 and 11 minutes respectively. When the same dose was given intravenously the average increases in blood pressures in these same animals were 23 and 27% with average durations of action of 15 minutes. Some respiratory embarrassment was seen following all such injections. In our experiments cardiac irregularities were frequently encountered in both cats and dogs. When the heart and lungs of these animals were excised and cut under water, droplets of oil were seen floating on the surface.

A third series of experiments was performed in an attempt to determine the volume of fluid retained in the bone marrow following an injection. In these experiments both posterior extremities of rabbits and cats were disjointed and severed from the body at the sacrum after the animals were dead. A cannula was inserted into the popliteal vein. India ink and mineral oil were injected into

the marrow cavity on alternate trials. Knowing the volumes held by the needle and cannula and the amount of fluid injected, it was possible to approximate the amount of the solution retained in the bone cavity. The average amount of either oil or india ink needed to fill that part of the marrow cavity which was injected was roughly for the rabbit 0.06 (25 observations) and for the cat 0.07 cc. (49 observations).

DISCUSSION. From the results of our experiments and those of Tocantins (2) and Tocantins and O'Neill (3) we believe that most water solutions (saline, glucose, blood plasma and blood), when injected into the marrow cavity, pass directly through the marrow spaces into the venous circulation and are immediately distributed throughout the body. Oils and suspensions of insoluble materials in water also pass directly into the general venous circulation but probably become lodged in the capillaries of the lungs before reaching the general arterial circulation. We were able to confirm Macht's (4) observations that when epinephrine in oil is injected into the bone marrow it causes a prolonged rise in blood pressure which was not seen on intramuscular injections. However, we

TABLE 1

	EPINEPHRINE		INCREASE IN BLOOD PRESSURE MM. HG	
	Dilution	cc. per kgm.	Drug Injected into	
			Vein	Bone marrow
Dog.....	1:10,000	0.1	63	47
	1:1,000	0.01	60	22
Cat.....	1:1,000,000	1.0	116	101
	1:100,000	0.1	102	35

observed the same circulatory effects when the epinephrine in oil was injected intravenously. According to Macht "Volatile oils are rapidly absorbed through the bone marrow but fixed oils are absorbed more slowly, thus retarding absorption of active principles dissolved therein." This difference in speed of action between essential oils and the fixed oils we believe to be due to the rapid volatilization of the essential oil in the blood stream and lung capillaries, thus freeing its drug for immediate action. When such oils are injected into the bone marrow of the tibia in 1 cc. doses, most of the injected oil passes through the marrow spaces and appears immediately in the popliteal and deep femoral veins. In those experiments which we performed with the use of these oils a fall in blood pressure was noted immediately upon their injection into the bone marrow. The reason for the slower "absorption" of chlorobutanol and camphorated oil, etc. dissolved in fixed oils is, we believe, that the drugs are liberated slowly from the droplets of oil lodged in lung capillaries and perhaps in the capillaries of other organs. We are convinced that the injection of any oily solution into the bone marrow is a dangerous procedure and should never be attempted in human beings nor

should irritants be so injected. Sterile saline, glucose, blood plasma, and blood can be injected by this route without danger to the patient. Because of the smallness of the veins leading from the bones all injections should be made slowly.

#### CONCLUSIONS

1. No gross difference in the latent periods and durations of action on blood pressure were noted between injections of epinephrine into veins and into bone marrow.
2. When small volumes of epinephrine are injected into the bone marrow the increase in blood pressure is approximately one-third that caused by a like intravenous injection. A small part of the injected material remains in the marrow spaces. When large volumes of a weaker solution are administered, most of the active substance passes quickly through the marrow spaces into the general circulation, consequently the increase in blood pressure is approximately equal to that induced by an intravenous injection.
3. Oils and suspended particles in water when injected into the marrow cavity of the tibial bone appear in the popliteal and deep femoral veins within one second after the beginning of the injection and they continue to be present as long as the injection lasts. A direct communication between the bone marrow and its venous drainage exists.
4. Because of the direct communication between the bone marrow and its venous drainage, oils, substances dissolved in oil and materials suspended in water should not be administered by this route.
5. Since a small amount of the injected material remains in the bone marrow irritant substances should not be administered by this route.

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# THE STIMULATING INFLUENCE OF SODIUM CITRATE ON CELULAR REGENERATION AND REPAIR IN THE KIDNEY INJURED BY URANIUM NITRATE<sup>1</sup>

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In 1916 MacNider (1) reported that sodium carbonate could be used to protect the dog's kidney against the toxic action of uranium nitrate. Later Goto (2) showed that sodium bicarbonate afforded the same type of protection against the substance. Whipple and his associates (3) found that hemoglobin gives protection for the kidney against injury by mercuric chloride. Recently Selye (4) made use of testosterone propionate to successfully protect the kidney of the mouse against mercuric chloride poisoning, and Kety and Letonoff (5) have reported the use of sodium citrate as an effective agent in the treatment of lead poisoning. There is no apparent correlation between these various factors which offer protection against heavy metal injury.

In this paper evidence is presented to show that sodium citrate leads to recovery following a lethal dose of uranium nitrate by facilitating repair and regeneration in the injured renal epithelium.

**METHODS.** Forty-six female dogs between the ages of one and three years, with weights varying between 5 and 12 kgm. were used in the experiments. All of the animals were maintained on a regular kennel diet of cooked meat scraps and uncooked bones, supplemented occasionally by Purina dog chow. The intake of water was not restricted.

Each animal received a single subcutaneous injection of uranium nitrate, 50 mgm. per kgm. of body weight in 0.5 per cent aqueous solution (Merck, lot S-7497). Prior to receiving uranium nitrate certain of the animals were given daily, for five successive days, intravenous injections through the jugular vein of a saturated aqueous solution of tri-sodium citrate 0.33 cc (approximately 230 mgm.) per kgm. of body weight. On the fifth day the uranium nitrate was injected in the amount and manner as before described. The intravenous tri-sodium citrate injections were then continued for five additional successive days following the uranium. The amount and timing of the citrate injections is explained by the fact that these studies grew out of other studies in which sodium citrate was used as an anti-coagulant (6).

Studies of the urine consisted of specific gravity determinations, qualitative tests for albumin, microscopic studies, and determination of phenolsulphonphthalein elimination. Studies of the blood were confined to determinations of non-protein nitrogen by the method of Folin and Wu (7) and of the carbon dioxide combining power of plasma by the method of Van Slyke and Cullen (8).

Anatomical studies were made on all animals which died, on certain animals which were sacrificed, and on survivors from which biopsy material was removed. Paraffin sections stained with hematoxylin and eosin were made routinely from the kidney and liver.

<sup>1</sup> Aided by funds from the Josiah Macy, Jr. Foundation and the John and Mary R. Markle Foundation.

**RESULTS.** In the first group of 13 control dogs which received one subcutaneous injection of 5 mgm. of uranium nitrate alone, all of the animals by the seventh day showed signs of a severe intoxication such as the refusal of food, nausea and vomiting, rapid loss of weight, inability to stand, and finally anuria. Between the ninth and thirteenth days 12 of the 13 animals died.

In the second group of 14 animals which received uranium nitrate preceded for 5 successive days and followed for 5 successive days by intravenous injections of 0.33 cc. per kgm. of a saturated solution of sodium citrate one animal died. The survivors in this group showed little, if any, clinical evidence of the intoxication. None of them failed to eat, and none of them showed any signs of nausea or weakness.

Albumin, red blood cells, and casts appeared in the urine of all animals within twenty four hours after injection of uranium. These changes persisted in the control group until an anuria developed, while in the "citrate" group the amount of albumin and the number of casts and red blood cells was diminished by the end of the second week and had largely disappeared by the end of the third week. Phenolsulphonphthalein elimination in both groups was reduced to virtually a negative reading and failed to increase in the control group. In the "citrate" group the output of the dye was increased to an average of 10% by the twelfth day and to 30% by the end of the third week.

The blood non-protein nitrogen in the control and "citrate" groups rose to comparable levels of retention during the first week. This level in the control group increased until death, while in the "citrate" group there was a definite decrease by the tenth or eleventh day. By the end of the third week the blood non-protein nitrogen in the animals of this group had returned to within normal limits.

The blood plasma carbon dioxide combining power showed about the same fall in the reserve alkali of both the control and "citrate" animals during the first week. By the ninth day, however, the "citrate" animals showed a rise toward normal which was completed by the end of the third week. In the control animals the diminished carbon dioxide combining power persisted until death.

Histological studies were made of the kidneys from a group of control and "citrate" animals sacrificed in pairs at intervals of 3, 6, 8, 10, and 12 days after the injection of uranium nitrate. In both groups, at the end of 3 days, the studies show the same apparent degree of injury with edema and early necrosis of the convoluted tubules especially of the proximal convolution. Figures 3 and 4 show comparable areas in the cortex of the kidneys of a control and of a "citrate" animal both three days after the commencement of the uranium intoxication.

In the 6 day animals histological studies show there is a more advanced injury with necrosis especially well seen in the proximal convolution of the tubule. Limited sloughing of the necrotic tissue has taken place and a flattened type of epithelium emphasized repeatedly by MacNider (9) is beginning to reline the necrotic tubules. At this stage these regenerative epithelial changes begin to be more prominent in the "citrate" animals. Figures 5 and 6 show comparable

areas in the cortex of the kidneys of a control animal and of a "citrate" animal both six days after uranium.

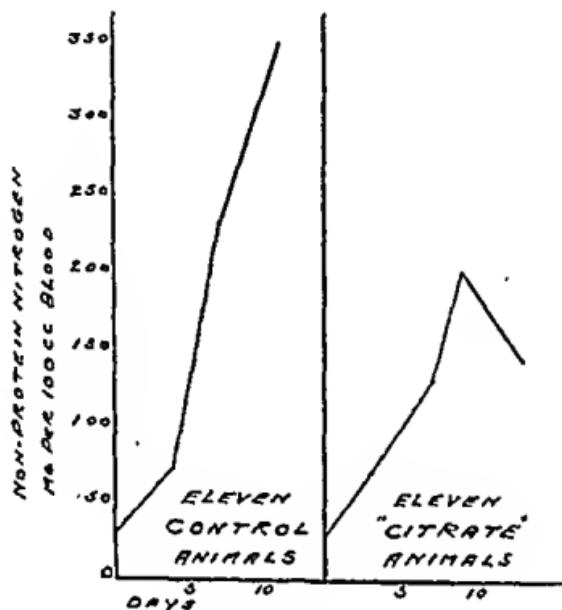


FIG. 1

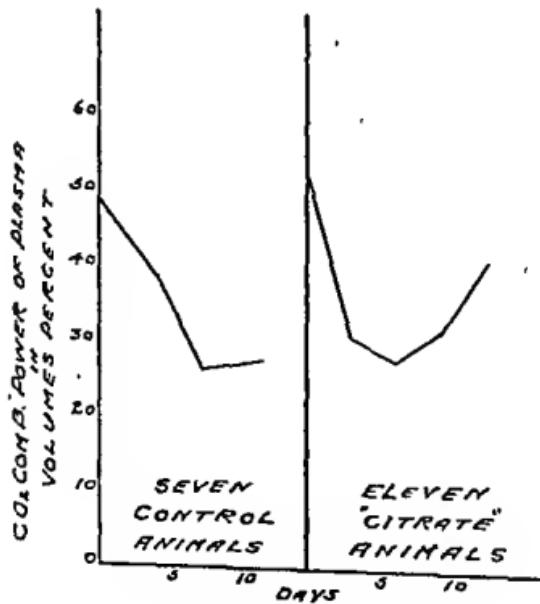


FIG. 2

The 12 day animals show by far the most striking difference seen in the histological studies. In the "citrate" animals approximately half of the tubules

# THE EFFECT OF SOME ANAESTHETIC AGENTS ON THE VOLUME OF BODY FLUID

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Scattered evidence exists that different anaesthetic agents produce alterations in the volume of blood and body fluids. McAllister (1) found in dogs that ether anaesthesia produced a decrease in plasma volume and an increase in red cell volume and plasma protein concentration. In humans, Stewart and Rourke (2) and Gibson and Branch (3) have reported that ether anaesthesia is followed by a decrease in plasma volume. Anaesthesia by barbiturates, on the other hand, appears to cause an increase in plasma volume (e.g. amyntal in dogs (Bollman *et al.* (4)), pentobarbital in cats (Hamlin and Gregersen (5)). These results explain the earlier observations of Hamburger and Ewing (6) and Searles (7) that ether in dogs caused an increase in red cell count and haemoglobin concentration, and of Barbour and Bourne (8), also in dogs, that the total blood solids were increased and of Searles (7) that amyntal in dogs caused a decrease in red cell count and haemoglobin concentration.

In view of these changes produced by anaesthetics, it has been thought that in the state of shock certain anaesthetics might be more harmful than others. Indeed, the opinion was widely held among military surgeons during the Great War 1914-1918 that ether and chloroform were more harmful to shocked patients than was nitrous oxide (Bazett (9), Krabbel (10)).

For many years the only experimental support for this view consisted of a few observations in cats by Dale (11). Recently, however, further support has been given by experiments on rabbits by Dafoe (12).

It seemed desirable, in this investigation, to extend the studies of the effect of various anaesthetics on blood volume to include nitrous oxide, cyclopropane and pentothal, and in addition to determine their effect on other components of the body fluid, in the hope of obtaining a clearer understanding of the effects of these agents on the normal circulation and possibly in shock.

**METHODS.** In this investigation the various body fluid compartments measured included plasma volume, and red cell volume, which together yield the total blood volume. In addition by the use of sodium thiocyanate, the total available fluid or thiocyanate space is determined, which is a hypothetical volume in which the thiocyanate is dissolved in a concentration equal to that of the plasma, and as used in this paper includes plasma and interstitial fluid, as defined below. Much evidence exists that thiocyanate is distributed among several compartments of the body fluid, such as plasma, red cells, extracellular fluid and certain intracellular spaces in gland cells. For purposes of this paper the extracellular fluid and the small portion of intracellular fluid entered by the thiocyanate are lumped together and termed interstitial fluid, and this volume is determined by the use of a formula presented later.

Plasma volume was measured by the use of the dye Evans Blue, T 1824, and total available fluid by means of sodium thiocyanate. These two materials were injected intra-

venously simultaneously (by a procedure similar to that described by Gregersen and Stew-  
ert (13)) and the concentration of the dye and thiocyanate in subsequent blood samples determined by the use of the Evelyn photoelectric colorimeter, using a G35 filter for the blue and a 490 filter for the thiocyanate.

Haemoglobin estimations were made using the method described by Evelyn (14). Red cell fraction estimations were carried out when each sample of blood was taken. Uniform glass tubing 0.5 mm. and 0.8 mm. in external diameter was used in 4-6 cm. lengths to obtain haematocrit values. After filling the tubes with blood, one end was sealed and they were spun for 5 minutes at 15,000-17,000 r.p.m. The value obtained from the haematocrit determination is called the per cent cell volume in this paper. At each time a blood sample was taken, duplicate haematocrit readings were made, and all the values obtained from the blood samples concerned in calculating the plasma volume were averaged to yield the per cent cell volume value for use in a calculation of the red cell volume.

*Procedure.* Male dogs varying in weight from 17-23 kgm. and trained to lie quietly on the operating table for 2-4 hours, were used in these experiments. Each animal was fasted 15-18 hours before being used and weighed before or after each experiment. Before commencing an experiment the animal was allowed to rest on the table 20-30 minutes. As the dogs weighed approximately the same, in each case 2 cc. of 1% Evans Blue and 8 cc. of 5% sodium thiocyanate solutions were used. The dye and thiocyanate solutions were measured out accurately into a beaker and the mixture taken up in a syringe, the beaker being rinsed with normal saline. Two such mixtures were prepared.

After taking a control sample of blood under oil from a leg vein, one of the dye mixtures was injected intravenously by the same vein, subsequent samples of blood were taken from a vein in the opposite leg at intervals of 30, 40 and 50 or 25, 35, 45 and 55 minutes. The drug or anaesthetic under investigation was now given and after a suitable interval for its action to develop, two or three blood samples were taken over the next 20-30 minutes. Then the second dye mixture was injected and at similar intervals to those used before, blood samples were taken.

It is realized that in this way there are two measures of the effect produced by any drug upon the fluid compartments measured, one obtained from alterations produced in the disappearance curves of the originally injected dye and thiocyanate, the other by the redetermination of the various volumes by use of the second dye mixture.

*Treatment of blood samples.* The blood samples were allowed to stand overnight in the ice chest. They were spun in the centrifuge, and the serum removed. Estimations of the dye and thiocyanate content of each were carried out on 0.5 cc. serum samples diluted to 6 cc. with 0.2% anhydrous sodium carbonate solution, as described below (a correction for any possible haemolysis which might have occurred that would affect the optical density of the Evans Blue was carried out. It was similar to that described by Evelyn (15)).

*Calculations.* The values for the Evans Blue and sodium thiocyanate of the various samples were plotted against time, and on extrapolation back to zero time a value is obtained which represents a certain amount of Evans Blue or sodium thiocyanate per cc. original serum as determined from the standard curves. As the amount of dye and thiocyanate injected is known, the value for plasma volume and total available fluid may be calculated.

The values for Evans Blue and sodium thiocyanate obtained after reinjection of the dye mixture were treated similarly. On extrapolation to zero time (the time of reinjection) a value is obtained, after which subtraction of the value existing at the time of reinjection yields a final value for the Evans Blue or thiocyanate which is treated as before, i.e. calculating the plasma or total available fluid (see fig. 1).

Red cell volume is calculated from the formula  $\frac{P.V. \times R}{S} = \text{red blood cell volume}$ , where  $P.V.$  = plasma volume,  $R$  = the average per cent red cell volume,  $S$  = the average per cent plasma volume. The total blood volume is the sum of the plasma volume and the red cell volume.

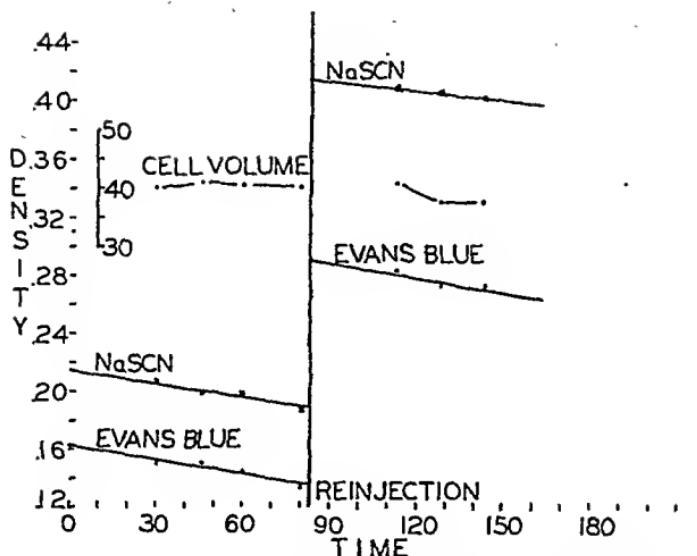


FIG. 1. THE PLOTTING OF A CONTROL EXPERIMENT, SHOWING THE CURVES FOR EVANS BLUE AND FOR THIOLYANATE; ALSO THE CHANGES IN PER CENT CELL VOLUME

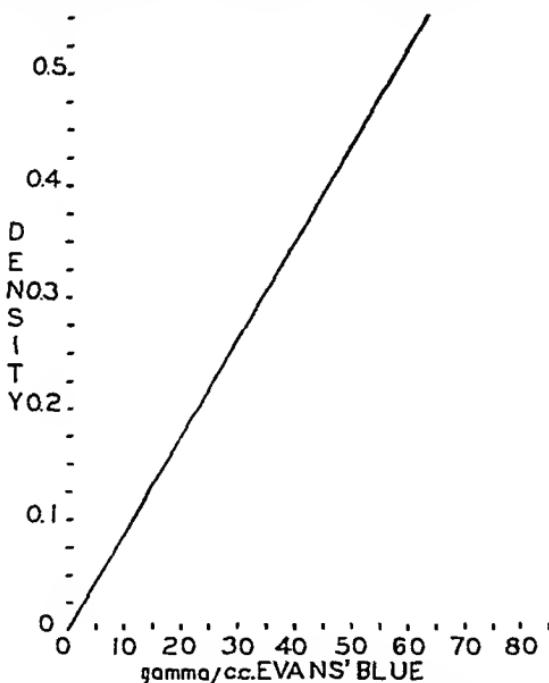


FIG. 2. THE STANDARD CURVE FOR EVANS BLUE

A criticism may be levelled at the method of calculating red cell volume; according to a number of workers the value arrived at in this manner is probably not the correct one (16), because the red cell fraction in capillaries is smaller than in larger vessels. On the other

hand, other investigators (17) have found a very close correlation between the values arrived at by this and the CO method. In this report changes in volume rather than absolute values are under consideration and so no correction for cell volume has been made.

The volume of interstitial fluid space is calculated by the formula  $I = 0.95 \left( \frac{Q}{S} - P - 0.5 C \right)$  where  $I$  = the interstitial fluid space,  $Q$  = the quantity of sodium thiocyanate injected, and  $S$  = the concentration of thiocyanate in serum,  $P$  = the plasma volume,  $0.5 C$  = a correction for the volume of fluid in red cells entered by thiocyanate,  $0.95$  = an arbitrary correction for the Donnan distribution between plasma and interstitial fluid.

*Chemical procedure.* Standard curves were prepared for each batch of Evans Blue and of sodium thiocyanate. To each of a series of calibrated colorimeter tubes was added 0.5 cc. serum and concentrations of Evans Blue, 5-50  $\mu$ gm., the final volume brought to 6 cc. with 0.2% anhydrous sodium carbonate solution. Using filter 635 in the colorimeter, the deflections produced by the various dilutions of Evans Blue were read from the scale. These values were transposed to density readings and plotted against the estimated known concentrations of the dye (fig. 2).

The standard curve for the thiocyanate was prepared similarly. To each of a series of calibrated tubes was added 0.5 cc. serum, concentrations of sodium thiocyanate varying from 20-100  $\mu$ gm., the final volume being brought to 6 cc. with the sodium carbonate solution. Then 1 cc. of 25% trichloracetic acid was added to each tube (this precipitated the protein and if any Evans Blue was present, it came down with the precipitate). After 5 minutes 1 cc. of the ferric nitrate reagent was added (Crandall and Anderson (18)) to each tube, the tubes were well shaken and allowed to stand for 5 minutes. They were then spun in the centrifuge for 10 minutes at 1300-1500 r.p.m., when they were shaken and respun for a further 10 minutes. This was found to yield very clear supernatants. Using filter 490 in the colorimeter the procedure was carried out as before, plotting the obtained density values against estimated known concentrations.

**EXPERIMENTS: NORMAL UNTREATED ANIMALS.** This series of experiments was undertaken to see how closely the various values determined by the initial injection of dye material checked with those obtained by the subsequent similar injection.

*Results.* Figure 1 is the plotting of one such experiment and the results obtained from 18 similar experiments are given in the table. No significant difference was found between the values obtained initially and those obtained after the injection of the second dye mixture for the plasma volume, red cell volume, total blood volume, total available fluid or calculated interstitial fluid space, while the second determination of percent cell volume showed a significant, though slight decrease.

Some experiments undertaken to determine the cause of this decrease in percent cell volume indicated that it was partly the result of further relaxation of the animal and partly due to the action of the thiocyanate. Even in control experiments a decrease in percent cell volume was observed, which reached a fairly constant level in about 30 minutes. These observations are similar to ones reported by Gibson and Evans (20) for humans, and by Hemingway, Scott and Wright (21) for haemoglobin values on repeated sampling in dogs.

The results of this series of experiments indicated that the method as employed would be suitable for the investigation of the effects of various drugs upon the volume of the different body fluid compartments.

EXAMINATION OF SOME PREMEDICANT DRUGS. *Experiments with atropine sulphate.* Doses of atropine sulphate (0.65-1.3 mgm.) sufficient to depress salivation occurring in ether anaesthesia, were given subcutaneously, after having determined the initial fluid volumes. The atropine was allowed to act for 35-40 minutes before redetermination of these various values was carried out.

*Results.* The results of the six experiments given in the table indicate that no significant changes occurred in any of the volumes measured.

*Experiments with morphine sulphate.* Doses of 30-35 mgm. of morphine sulphate in a 4% solution were given subcutaneously after initial determination of the volumes. These amounts of morphine produced a quieting effect upon

TABLE 1

The differences between the initially determined and redetermined volumes in each case are expressed as percentage change of the initial value. The mean of these differences for each case is calculated and the statistical significance determined by the use of Fisher's "T" test (19). The significant values in the table are marked with an asterisk.

SERIES	NO. OF CASES	PLASMA VOLUME	RED CELL VOLUME	TOTAL BLOOD VOLUME	TOTAL AVAILABLE FLUID	CALCULATED INTERSTITIAL FLUID SPACE	PER CENT CELL VOLUME
Untreated.....	18	+0.7	-1.0	-0.2	-0.1	-0.2	-1.4*
Atropine.....	6	+1.6	+0.5	+1.1	-1.2	-1.7	-0.7
Morphine.....	8	-2.6	+53.8*	+20.1*	+1.1	-2.3	+27.3*
Morphine and atropine.....	8	+0.5	+15.9*	+6.6*	-8.1*	-12.2*	+8.5*
Ether.....	10	-5.4*	+58.3*	+22.2*	-10.4*	-17.3*	+30.4*
Morphine, atropine and ether .....	8	-9.0*	+56.9*	+17.1*	-11.6*	-17.3*	+33.3*
Pentobarbital.....	10	+8.9*	-11.3*	+0.3	-1.3	-2.6	-11.5*
Pentothal.....	6	+10.2*	-8.2	+2.3	-7.1	-10.2	-10.5*
Nitrous oxide.....	5	+3.2	+52.2*	+24.8*	+0.6	-3.9	+21.6*
Cyclopropane.....	5	-2.5	+56.4*	+22.4*	+3.0	+0.7	+27.3*

the animals. Thirty-five to 40 minutes were allowed for the action of the drug to develop before redetermining the various values.

*Results.* The results obtained from 8 such experiments are shown in the table and it is seen that no significant changes were observed in the plasma volume and total available fluid or calculated interstitial fluid space. The per cent cell volume showed an increase of 27.3%, the red cell volume an increase of 53.8% and the total blood volume an increase of 20.1%. The changes in haemoglobin values followed those of per cent cell volume closely. In similar experiments carried out with 30 mgm. doses of morphine on a splenectomized animal, the only change noted was a slight decrease in per cent cell volume and haemoglobin after the administration of morphine.

The increase in per cent cell volume is probably a little greater than the figures indicate, for in the control series the per cent cell volume showed a slight, but significant, decrease after the second injection of dye mixture. Since the

plasma volume was unchanged, the increases in haemoglobin and in per cent cell volume must be due chiefly to an actual increase in the number of circulating red cells. It was thought that this increase, and the resulting rise in blood volume, was due to splenic contraction and this is borne out by the experiments on a splenectomized animal.

On one human subject it was shown that morphine in therapeutic doses, 16 mgm. and 21.6 mgm. produced some increase in per cent cell volume, the small dose producing a 3% increase, the larger one a 6% increase. In the case of the larger dose more than 20 minutes were required to elicit the maximum response.

*Experiments with morphine and atropine sulphate.* Doses of morphine ranging from 16-32 mgm. and doses of atropine of 0.65 mgm. were injected subcutaneously after the initial fluid volume determination. This mixture of drugs was allowed to act for 30-40 minutes and then the procedure was carried out as before.

*Results.* The table shows the results obtained from experiments using 5 different animals.

Plasma volume showed no significant change, while there was a significant decrease in the total available fluid of 8.1% and of 12.2% in interstitial fluid. The red cell volume showed an increase of 15.9%, the total blood volume an increase of 6.6% and the per cent cell volume an increase of 8.5%. Haemoglobin values followed the changes in per cent cell volume closely.

From the changes in the disappearance curve of the thiocyanate prior to redetermination, and after giving the morphine and atropine, variable results were obtained. The average was a decrease of 2.5% (5 showed a decrease, 2 an increase and one no change).

With the exception of the decrease in total available fluid, the results look very similar to those obtained with morphine alone, save that the increases in red cell volume, per cent cell volume and total blood volume are of less degree. This is probably due to the fact that 5 of these experiments were carried out with much smaller doses of morphine. The changes in red cell volume, blood volume and per cent cell volume must be due to the same mechanism, *i.e.* splenic contraction, which accounted for changes when morphine alone was used. It is of interest that Ragan and co-workers (22) observed no change in plasma volume, as determined by T 1824, in a group of patients, as a result of morphine and atropine premedication.

The significant decrease in the total available fluid is remarkable in that no such change was observed when the drugs were examined separately. The mechanism for the production of this change is uncertain, but since the interstitial fluid space was decreased and no change was observed in plasma volume, it might be suggested that fluid passed from the interstitial to the intracellular space.

**EXAMINATION OF SOME ANAESTHETIC AGENTS.** *Experiments with ether.* After completion of the initial determination of the various fluid compartments, the dogs were anaesthetized in an ether box. Anaesthesia was maintained 30-45 minutes before redetermination of the various values was carried out.

**EXAMINATION OF SOME PREMEDICANT DRUGS.** *Experiments with atropine sulphate.* Doses of atropine sulphate (0.65-1.3 mgm.) sufficient to depress salivation occurring in ether anaesthesia, were given subcutaneously, after having determined the initial fluid volumes. The atropine was allowed to act for 35-40 minutes before redetermination of these various values was carried out.

**Results.** The results of the six experiments given in the table indicate that no significant changes occurred in any of the volumes measured.

*Experiments with morphine sulphate.* Doses of 30-35 mgm. of morphine sulphate in a 4% solution were given subcutaneously after initial determination of the volumes. These amounts of morphine produced a quieting effect upon

TABLE 1

The differences between the initially determined and redetermined volumes in each case are expressed as percentage change of the initial value. The mean of these differences for each case is calculated and the statistical significance determined by the use of Fisher's "T" test (19). The significant values in the table are marked with an asterisk.

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Atropine.....	6	+1.6	+0.5	+1.1	-1.2	-1.7	-0.7
Morphine.....	8	-2.6	+53.8*	+20.1*	+1.1	-2.3	+27.3*
Morphine and atropine.....	8	+0.5	+15.9*	+6.6*	-8.1*	-12.2*	+8.5*
Ether.....	10	-5.4*	+58.3*	+22.2*	-10.4*	-17.3*	+30.4*
Morphine, atropine and ether.....	8	-9.0*	+56.9*	+17.1*	-11.6*	-17.3*	+33.3*
Pentobarbital.....	10	+8.9*	-11.3*	+0.3	-1.3	-2.6	-11.5*
Pentothal.....	6	+10.2*	-8.2	+2.3	-7.1	-10.2	-10.5*
Nitrous oxide.....	5	+3.2	+52.2*	+24.8*	+0.6	-3.9	+21.6*
Cyclopropane.....	5	-2.5	+56.4*	+22.4*	+3.0	+0.7	+27.3*

the animals. Thirty-five to 40 minutes were allowed for the action of the drug to develop before redetermining the various values.

**Results.** The results obtained from 8 such experiments are shown in the table and it is seen that no significant changes were observed in the plasma volume and total available fluid or calculated interstitial fluid space. The per cent cell volume showed an increase of 27.3%, the red cell volume an increase of 53.8% and the total blood volume an increase of 20.1%. The changes in haemoglobin values followed those of per cent cell volume closely. In similar experiments carried out with 30 mgm. doses of morphine on a splenectomized animal, the only change noted was a slight decrease in per cent cell volume and haemoglobin after the administration of morphine.

The increase in per cent cell volume is probably a little greater than the figures indicate, for in the control series the per cent cell volume showed a slight, but significant, decrease after the second injection of dye mixture. Since the

of an altered or deranged metabolism, and it may well be that the decrease observed here in interstitial and total available fluids is yet another indication of this change.

*Experiments with morphine, atropine and ether.* This series of investigations was undertaken to see if adequate premedication would modify the effects produced by ether.

After determining the initial volumes, morphine in doses of 16-40 mgm. and atropine (0.65 mgm.) were given subcutaneously and allowed 30-40 minutes to exert their action. Then ether anaesthesia was induced, using a mask and dropping hoffle. Anaesthesia was maintained 25-35 minutes before redetermining the various fluid volumes.

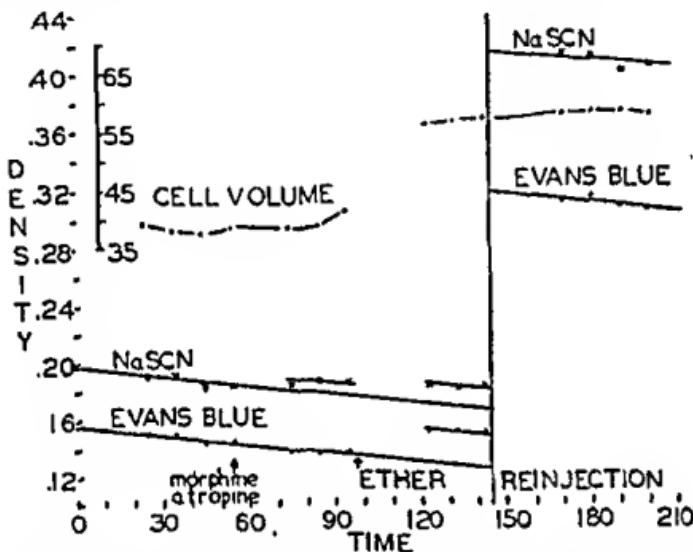


FIG. 3. THE PLOTTING OF THE EVANS BLUE, THIOCYANATE AND PER CENT CELL VOLUME VALUES FROM AN EXPERIMENT WHEN MORPHINE AND ATROPINE WERE GIVEN PRIOR TO THE PRODUCTION OF ETHER ANAESTHESIA

*Results.* The results of eight experiments using five animals are given in the table and figure 3 shows the plotting of such an experiment. The plasma volume showed a decrease of 9%, the total available fluid a decrease of 11.6% and the interstitial fluid volume a decrease of 17.3%, these being significant. Significant increases were observed in the per cent cell volume of 33.3%, in the red cell volume of 56.9% and in total blood volume of 17.1%.

The disappearance curve of Evans Blue was unaffected by the morphine and atropine, but after induction of anaesthesia there was an increase in the concentration of the dye; the average decrease in plasma volume calculated from this change was 12%. The disappearance curve of the thiocyanate exhibited a concentration following the injection of morphine and atropine, with a somewhat greater concentration after the induction of ether anaesthesia. The decrease in total available fluid calculated from this change was 10.6%.

The changes observed here are very similar to those seen in the ether series. The decrease in plasma volume is somewhat greater, and would account for some 20-30% of the total increase in per cent cell volume. As before, there is an increase in the number of circulating red cells resulting in an increased total blood volume. This increase in blood volume is less than in the previous series and is probably related to the greater decrease in plasma volume.

Since salivation was abolished by the action of atropine and the same changes in plasma volume and total available fluid occurred as when there was marked salivation, it seems clear that salivation must have exerted little, if any, effect on these changes.

The changes in total available fluid and interstitial fluid space are most probably due to the same mechanism as when ether alone was used.

*Experiments with pentobarbital.* After initially determining the various fluid volume values, anaesthesia was produced by an intravenous injection of 22-24 mgm. per kgm. of a 10% solution of pentobarbital sodium. Injection was made slowly so that no respiratory arrest was produced. After anaesthesia had persisted for 25-40 minutes, redetermination of the various fluid compartments was carried out.

*Results.* The table presents the results obtained in ten experiments. It is seen that there was a significant increase of 8.9% in plasma volume, while per cent cell volume shows a decrease of 11.5% and red cell volume of 11.3%. Haemoglobin values followed the changes in per cent cell volume. No significant alterations were observed in the total blood volume, the total available fluid or the interstitial fluid space.

The disappearance curve of Evans Blue prior to redetermination exhibited a decrease in concentration as a result of anaesthesia in all but one case, where no change was observed. The average increase in plasma volume as a result of this change was 11.2%.

The increase in plasma volume is sufficient to account for some 60-70% of the decrease in per cent cell volume; the rest of the decrease must result from removal of cells from the circulation. The spleen may be suggested as the reservoir, and indeed Hausner, Essex and Mann (24) have observed the spleens of dogs anaesthetised with pentobarbital, amytal and with pentothal, to be enlarged.

Hamlin and Gregersen (5) report that pentobarbital anaesthesia produces a similar increase in plasma volume in cats, while on the other hand Gibson and Branch (3) state that a decrease in plasma volume was observed in dogs, but no figures are given.

Nowak (26) and Tournade and Joltrain (27) have reported that some barbiturates produce some vasodilation, but as blood pressure remains good it cannot play any important role in the increase in plasma volume. It is interesting that the interstitial fluid space shows a decrease of the same order of magnitude as the increase in plasma volume, the average figures being 123 and 101 cc. respectively. This suggests that the increase in plasma volume is made at the expense of the interstitial fluid.

In one of the experiments where a period of 130 minutes passed before redetermination was carried out, a greater increase in plasma volume was observed shortly after induction of anaesthesia than was present later on when the dog was beginning to show signs of lightening of anaesthetic depth. If this occurs in other pentobarbital experiments, it would account for the larger figures for plasma volume increase being obtained from the disappearance curve of the dye before redetermination of the plasma volume was carried out.

*Experiments with pentothal sodium.* Anaesthesia was induced after the initial determination of the various fluid compartments by the intravenous injection of a 10% solution of pentothal. The amount used varied from animal to animal and it was found necessary to give further small injections of this solution throughout the course of the experiment to maintain anaesthesia at a fairly constant level. After 30-40 minutes of anaesthesia, redetermination of the various volumes was carried out.

*Results.* The table presents the results of 6 experiments using 3 animals. It is seen that there was a significant increase in plasma volume of 10.2% and a decrease in per cent cell volume of 10.5%. No significant variation was observed in red cell volume, blood volume, total available fluid or calculated interstitial fluid space.

The disappearance curves of Evans Blue prior to redetermination show a decrease in dye concentration, the average increase in plasma volume being 6%.

The increase in plasma volume accounts for some 80-90% of the decrease observed in per cent cell volume. One must assume that some cells have been removed from the circulation and it was noted before (24) that the spleen was enlarged during pentothal anaesthesia. This would suggest that there should be a decrease in red cell volume, which would be significant, and it is possible that in a larger series of experiments the value for this volume would assume a significant character.

The changes in total available fluid in all dogs prior to redetermination were in a negative direction, while all but one also showed a decrease by redetermination; and in a larger series with more animals the values for the changes in total available and interstitial fluid might be significant.

It is interesting that Searles (7), Searles and Essex (7) and Bollman (4) have reported changes in plasma volume, red cell volume and total blood volume as a result of amyta anaesthesia similar to those observed here as a result of pentothal and of pentobarbital anaesthesia.

*Experiments with nitrous oxide and oxygen.* It was necessary to use morphine and atropine as premedicants prior to producing anaesthesia with nitrous oxide and oxygen. Only one of the animals was deeply anaesthetized, while the other two animals were in the first plane of the third stage of anaesthesia during most of the time of experiment.

Doses of morphine ranging from 45-65 mgm. and atropine in 0.65 mgm. doses were given subcutaneously. These premedicants were allowed to act for 35-40 minutes before the establishment of anaesthesia and after a period of 20-35 minutes anaesthesia the procedure was carried out as before.

*Results.* The results from five experiments are shown in the table and it is seen that no significant changes occurred in plasma volume, total available fluid or interstitial fluid space. The disappearance curve of both the Evans Blue and the thiocyanate prior to redetermination yielded a similar result. Significant increases were observed in the per cent cell volume of 21.6%, in red cell volume of 52.2% and in blood volume of 24.8%. The variation in haemoglobin followed the changes in per cent cell volume fairly closely.

In each case the morphine used as a premedicant produced an increase in per cent cell volume, and in three of the experiments this increase was equal to or greater than the increase observed after the establishment of anaesthesia.

Since the plasma volume was unchanged and the changes in haemoglobin values followed those in per cent cell volume, there must have been an actual increase in the number of circulating red cells. The source of these cells is most probably the spleen. Hamburger and Ewing (6) have reported increase in red cell count, haemoglobin and per cent cell volume following anaesthesia with nitrous oxide in dogs.

*Experiments with cyclopropane.* After some initial experiments it was found necessary to use atropine (0.65 mgm.) as a premedicant to control salivation, for a closed system was used. Otherwise the procedure was carried out as before, allowing a period of 25-40 minutes to elapse between the induction of anaesthesia and remeasurement.

*Results.* The table presents the results of 5 experiments in which atropine was used as a premedicant. As in the experiments with nitrous oxide, the only significant changes were an increase in per cent cell volume of 27.3%, in red cell volume of 56.4% and in total blood volume of 22.4%. Haemoglobin values followed the changes in per cent cell volume fairly closely.

From the disappearance curve of the Evans Blue prior to redetermination in all cyclopropane experiments, there was an increase in the concentration of the dye, yielding on the average a decrease in plasma volume of 4.4%, while no significant alterations were observed in the thiocyanate disappearance curves.

In view of the decrease in plasma volume observed prior to redetermination, it is possible that in a larger series of experiments with more animals the change in plasma volume obtained by remeasurement might assume significance.

Considering the changes obtained by redetermination, the picture is similar to that observed in the nitrous oxide series. The increase in circulating red cells results in an increased blood volume, the spleen being suggested as the source of these cells.

#### SUMMARY

The evidence presented above certainly suggests that ether, even with morphine and atropine premedication, would not be advisable in the case of shock, owing to the fact that plasma volume is decreased and also that there is a decrease in interstitial fluid which may be secondary to the deranged cellular metabolism.

The evidence for both nitrous oxide and cyclopropane appears much more favourable. Both the barbiturates increased plasma volume with little change in blood volume, yet their value is more difficult to assess as the decrease in interstitial fluid was not significant.

*Acknowledgement.* Appreciation is expressed to Prof. V. E. Henderson and to Prof. J. K. W. Ferguson for their criticism and advice.

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## ANESTHESIA

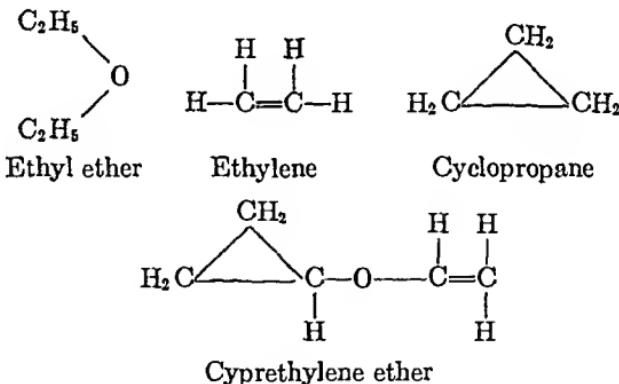
### VI. THE ANESTHETIC ACTION OF CYCLOPROPYL VINYL ETHER<sup>1</sup>

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In former communications (1 and 2) the authors reported their findings on the anesthetic properties of cyclopropyl methyl ether and cyclopropyl ethyl ether. Studies with the methyl homolog (cyprome ether) in man have been published (3); those with the ethyl homolog (cypreth ether) will be published shortly. In the synthesis of this series of cyclopropyl aliphatic ethers, it was considered of interest to prepare the unsaturated analog of cypreth ether. This compound has in its molecule, first, the cyclopropyl group, second, the carbon-oxygen-carbon linkage of ethyl ether, and third, the double bond of ethylene. In this laboratory the compound has been called "cyprethylene ether." Its relationship to ether, cyclopropane and ethylene is apparent from the following formulas:



Cyprethylene ether is a volatile, colorless liquid with a characteristic etherial odor; it boils at 67°C. and has a specific gravity of 0.833 at 25°C.

*Anesthesia in the monkey.* Five large Rhesus monkeys were each anesthetized twice with cyprethylene ether. The technic is described in detail in our previous communication (1). The induction period was of short duration; frequently the monkeys passed into the plane of surgical anesthesia without appreciable struggle. During induction, salivation was not marked and the bronchial tree remained quite free from mucus throughout the anesthesia. Surgical anesthesia was uneventful. Breathing was often stertorous but deep and regular. Relaxation of the musculature of the abdomen and extremities was complete under

<sup>1</sup> The expense of this investigation was defrayed in part by a grant from the Ohio Chemical and Manufacturing Company of Cleveland, Ohio.

very light anesthesia. Only an occasional animal exhibited incoordinated leg movements during anesthesia. These occurred near respiratory collapse and were perhaps due to anoxia. Pain reflexes were abolished, even when the anesthesia was light. Recovery from anesthetics of 30 minutes duration was very prompt, resembling the rapidity of recovery from a divinyl oxide anesthesia. There was little excitation during the recovery period. The quantities of cyprethylene ether employed were approximately one-third the amounts of ethyl ether required for similar anesthetic syndromes.

TABLE I  
Anesthetic Index

DOG NUMBER	SEX	WEIGHT kgm.	INDUCTION cc./kgm.	RESPIRATORY FAILURE cc./kgm.	ANESTHETIC INDEX
1	F	6.1	0.33	1.97	5.97
2	M	0.4	0.32	1.76	5.50
3	F	5.8	0.35	1.12	3.20
4	F	11.5	0.31	1.44	4.65
5	F	6.3	0.32	1.52	4.75
6	M	9.6	0.26	1.31	5.04
7	F	6.0	0.33	1.17	3.55
8	M	0.2	0.33	1.79	5.43
9	M	9.6	0.26	1.15	4.42
10	M	9.7	0.26	0.98	3.77
11	F	6.5	0.39	1.62	4.15
12	F	10.0	0.30	1.30	4.33
13	F	6.1	0.41	1.31	3.20
14	F	6.1	0.41	1.88	4.59
15	F	6.6	0.38	1.75	4.60
16	M	8.0	0.33	2.00	5.26
17	M	9.1	0.33	1.38	4.18
18	M	9.5	0.32	1.16	3.63
Mean			0.33	1.48	4.42
$\sigma$			0.017	0.32	0.79
C.V...			14.2	21.6	17.9

*Anesthetic index (dog).* The dogs were fed a standard laboratory ration consisting mainly of "Purina Chow" during 1 week and fasted 12 hours before anesthesia. At least 2 day intervals elapsed between anesthetics in the same animal. The procedure employed was identical with that used in the cyprome and cypreth ether studies. The number of cubic centimeters of the anesthetic agent per kilogram required to produce surgical anesthesia was divided into the volume per kilogram required to produce respiratory arrest and the quotient designated as the anesthetic index. The results are summarized in table 1.

In this series of 18 anesthetics, only one animal succumbed; all others were revived by artificial respiration. In our previous communication (3) for comparative purposes experiments were conducted with ethyl ether, cyprome ether,

divinyl oxide and chloroform using the same technic. Making all allowances for the difference in densities, the potency of cyprethylene ether approaches that of chloroform. It appears to be slightly more potent than divinyl oxide. The difference between the induction dose and respiratory arrest dose of ethyl ether is 1 cc. per kgm., with cyprethylene ether it is 1.15 cc. per kgm.

*Blood pressure studies (dog).* The effect of cyprethylene ether on the blood pressure was determined by cannulating the femoral artery under procaine hydrochloride anesthesia and preparing the animal for a blood pressure tracing in the usual manner. The respiratory tracings were made by means of a chest tambour. After a normal tracing, cyprethylene ether was administered by the

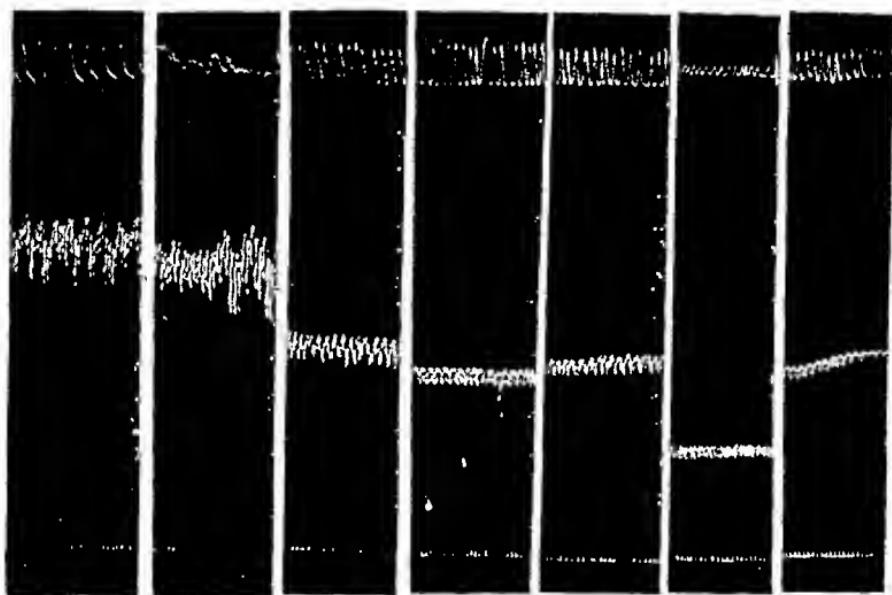


CHART 1. BLOOD PRESSURE OF THE DOG UNDER CYPRETHYLENE ETHER ANESTHESIA

The upper tracing is respiration. (1) is normal, (2) is the induction period, (3) surgical anesthesia after 10 minutes, (4) surgical anesthesia after 40 minutes, (5) surgical anesthesia after 60 minutes, (6) threatened respiratory collapse, (7) recovery 5 minutes later.

same technic employed to measure the anesthetic index. In four experiments respiratory failure occurred before cardiac stoppage. The blood pressure was depressed slightly before the onset of surgical anesthesia and deeply at the point of threatened collapse. Animals tested in the same manner, but the amount of anesthetic used was increased to the various stages of anesthesia. Portions of the data are given in Table 1.

*Electrocardiographi-  
thetized with cypret  
Just prior to the ex-  
periment on the electro-  
cardiogram of the anesthe-  
tized animals.*

regularity of the E.C.G. The fourth animal, during light anesthesia, exhibited a marked increase in the rate concomitant with a left bundle branch block which varied in degree; there was also some slurring of the *S*-wave. The duration of the *R*-spike was increased considerably. These abnormalities were evident in all three leads, but were most marked in Lead III. The sixth animal in the group, in which the two stages of anesthesia were definitely observed, showed in the induction period some extraventricular systoles in Lead III; there were cyclic changes in the amplitude of the *R* and *S* waves. There was also some evidence of partial bundle branch block. These abnormalities disappeared upon the onset of surgical anesthesia. Changes found in the induction stages in the E.C.G. of these two monkeys, at the time of writing (20 anesthesias) have not been observed in man with the stethoscope. We wish to caution anyone, however, who administers this compound to man while these studies are still in their early stages. Electrocardiograph studies in human beings will be conducted.



CHART 2. ELECTROCARDIOGRAMS, NORMAL AND UNDER CYPRETHYLENE ETHER ANESTHESIA

*Effect on the perfused heart (frog).* Cyprethylene ether was dissolved in Howell-Ringer's solution and perfused through the frog's heart *in situ*. Several dilutions were employed to determine the threshold concentration required to cause demonstrable changes within one minute. In addition, the concentration of cyprethylene ether found in the monkey's blood during surgical anesthesia (approximately 25 mgm. per cent or 0.003 molar) was perfused repeatedly for long periods of time. A typical tracing is shown in chart 3. The threshold concentration of cyprethylene ether required to produce cardiac effects in 5 minutes was 0.0045 molar. The tracing shows the 0.003 molar cyprethylene ether perfusion. In 7 animals, 0.003 molar cyprethylene ether produced slight diminution in rate and amplitude of heart as a typical effect. Occasionally after long perfusion an extra systole was observed.

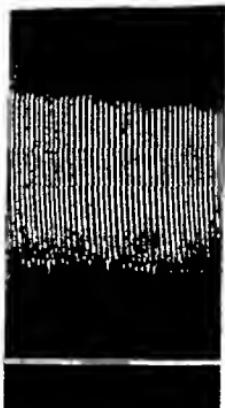
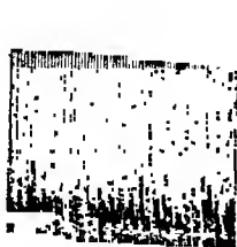
*Liver function tests (monkey).* Seven monkeys were subjected to the bromsulphthalein liver function test as set forth in the previous studies (1, 2). Also the test was repeated 3 times increasing the dye excretion period to 30 minutes.

\* We are indebted to Dr. Robert H. Oster of the Department of Physiology for his assistance in conducting this study and for his advice in interpreting the findings.

Twenty-four hours after a 30 minute anesthesia with cyclopropane ether the dye excreted in all cases was not significantly different from the preanesthetic excretion rate.

*Blood chemistry studies (monkey).* Rhesus monkeys were anesthetized to the surgical plane for 15 minutes with cyprethylene ether. Prior to anesthesia and 24 hours later blood samples were drawn for analysis. No significant changes in carbon dioxide combining power or urea nitrogen were observed.

*Delayed anesthetic deaths (rat).* Twenty-one male adult rats were anesthetized with cyprethylene ether to the surgical plane and maintained in this condition for one-half hour. Immediately afterwards one animal was sacrificed and its liver and kidneys examined histologically. No abnormalities were found. Four additional animals were studied in the same manner and after two weeks no significant findings were observed. At the end of three weeks none of the remaining animals had died or appeared to be in an unhealthy condition.



*Concentration required for anesthesia (mouse).* The concentration of cyprethylene ether required to induce anesthesia was determined by typical partial pressure experiments described previously (1). The results are shown in table 2. With ethyl ether, in our previous studies, 4 per cent partial pressure produced no anesthesias and 5 per cent anesthetized 40 per cent of the animals.

*Histological studies of viscera (rat, mouse, dog and monkey).* Five of the rats used in the delayed anesthetic death experiments were sacrificed for histological study. Their lungs, livers, spleen and kidneys were found to be free from significant changes. Three of the mice used in the partial pressure experiments were sacrificed and their livers, lungs and kidneys examined histologically. The findings were negative. Five dogs were anesthetized lightly with cyclopropane and liver biopsies performed. The anesthetic agent was changed to cyprethylene ether and maintained at the surgical level for one hour and a second biopsy performed. There were no significant pathological findings. Five Rhesus

monkeys were subjected to the foregoing biopsy procedure as performed on the dogs. The findings in this series coincide with those described with the dog anesthesias.

*Clotting time and hemolysis (monkey and dog).* The clotting time of blood was determined in 3 normal Rhesus monkeys by the capillary tube method. The average clotting time was approximately one minute. Within the error of the experiment this period was neither diminished nor increased under surgical anesthesia with cyprethylene ether.

Volumes of 20 cc. of cyprethylene ether in varying concentrations in normal salt solution were maintained at 37°C. To each of these was added 0.1 cc. of freshly drawn defibrinated dog's blood and the time required for hemolysis was observed. Solutions of cyprethylene ether 0.003 molar or 25 mg. per cent, produced no hemolysis within a 4 hour period of observation at 37°C. Solutions 0.013 and 0.025 molar respectively produced no hemolysis over a 6 hour observation period at 25°C., however, 0.05 molar solutions produced hemolysis within one and one half hours.

TABLE 2  
Induction Concentration in Mice

PARTIAL PRESSURE, PER CENT	CC. PER LITER	NO. MICE PER JAR	NO. MICE USED	PER CENT ANESTHETIZED
0.5	0.02	5	5	0
1.0	0.04	5	5	0
1.5	0.06	3	3	0
2.0	0.08	5	16	31
2.25	0.09	5	20	50
2.50	1.00	5	36	95
3.00	1.20	5	35	98

*Irritation of mucous membranes.* The late Dr. Karl Coanell tested the vapor of cyprethylene ether on his conjunctiva repeatedly on successive days. He compared various partial pressures with those of other anesthetics. From his vast experience with the vapor of ethyl ether the reproducibility of his findings was excellent. On a comparative basis in the ascending order of irritation he considered the irritating properties of the vapors to fall in the following order: divinyl oxide 1, cyprethylene ether 2, ethyl ether 7, and cypreth ether 12.

*Preanesthetic medication (monkey and dog).* With monkeys and dogs morphine-atropine medication influenced the anesthesia with cyprethylene ether in the same manner which it affects the anesthesia of ethyl ether. In monkeys, inducing cyprethylene ether anesthesia with nitrous oxide-oxygen or cyclopropane-oxygen mixtures was uneventful. Preanesthetic medication with pentobarbital sodium was found to be entirely compatible with cyprethylene ether anesthesia. Ten experiments in all were conducted.

*Quantitative determination in blood (dog).* The method used by Andrews *et al* (4) for the determination of ethyl ether in the blood was employed with certain

minor modifications. The method essentially consists of aspirating the cyprethylene ether from the blood by means of air into standard potassium dichromate solution in 7 molar sulfuric acid. The excess of oxidizing agent is determined iodometrically. Recoveries from blood of known cyprethylene ether content were found to be complete. Five determinations were made on monkey's blood under surgical anesthesias of about 10 minutes duration; the mean value in milligrams per cent was 22.3 (range 26.0 to 19.9).

*Physical properties.* *Solubility in water.* Five cc. of cyprethylene ether were mechanically agitated with 100 cc. of water for 3 hours at 27°C. in a "Cassia Flask." The two liquids were allowed to separate for 12 hours and the volume of the supernatant cyprethylene ether was measured. The solubility of cyprethylene ether was found to be 0.8 cc. per 100 cc. of water. The molar concentration of a saturated solution of cyprethylene ether in water is 0.08 at 27°C.

*Oil/water coefficient.* A 0.1 molar concentration of cyprethylene ether in corn oil (free fatty acids less than 0.03 per cent) was prepared. The oil/water coefficient was determined by the procedure of Cone *et al.* (5). The molar concentration in the water at equilibrium (5 experiments) was found to be 0.0015. The oil/water coefficient of cyprethylene ether measured under these conditions is 67.

*Inflammability range.* Mixtures of cyprethylene ether vapor and oxygen were prepared at 25°C. and at atmospheric pressure in an explosion pipette. The mixtures were exposed to the spark of an induction coil. Explosions occurred when the concentration of cyprethylene ether mixed with oxygen was 1.9 per cent. Concentrations below this value could not be exploded under these conditions.

*Vapor pressure.* The vapor pressure of cyprethylene ether at 24°C. determined in a nitrometer, was 160 mm., that of ethyl ether at the same temperature is 510 mm. (6).

#### SUMMARY AND CONCLUSIONS

1. The union of the molecule of cyclopropane through an ether linkage with the vinyl radical results in the formation of a volatile liquid ("Cyprethylene Ether") exhibiting anesthetic properties when administered by inhalation to various species of animals.

2. Cyprethylene ether with the characteristic structures of ethyl ether, cyclopropane and ethylene exhibits a potency which approximates chloroform and an anesthetic index more than twice that of ethyl ether.

3. In the monkey, cyprethylene ether produces no functional liver damage as shown by the bromsulphthalein test. In these experiments, in the mouse, rat, dog and monkey anesthesias with cyprethylene ether produced no significant histopathological changes in the important viscera.

4. The monkey's heart under surgical anesthesia, as a rule, showed no abnormalities. In the induction stage the irregularities experienced are discussed in the text. The frog's heart was slightly depressed in rate of beat and amplitude when perfused with anesthetic concentrations of cyprethylene ether.

5. The explosive range of concentrations of cyprethylene ether with oxygen appears to be approximately the same as that of ethyl ether. The oil/water coefficient of cyprethylene ether is approximately 16 times greater than that of ethyl ether. Anesthetic concentrations in the blood are approximately one-fifth of those found with ethyl ether.

6. Cyprethylene ether boils at 67°C.

7. The blood pressure is only slightly lowered by anesthetic concentrations of cyprethylene ether in the dog.

8. This first approximation of the pharmacology of cyprethylene ether, in our opinion, warrants its careful and judicious trial in man by skilled anesthetists.

*Addendum.* These experiments having been completed, we deemed that the properties of cyprethylene ether warranted trial as an anesthetic in man. In November Dr. Melvin G. Kilborne of West Orange, N. J., administered cyprethylene ether to man for the first time. In all, 20 human anesthesias have been given up to the time of writing without any untoward reactions. A complete series of cases will appear as a subsequent communication.

*Acknowledgements.* We wish to express our appreciation of and bereavement at the loss of Dr. Edith F. Sollers who was killed in an explosion in the preliminary synthesis of this compound.

We are indebted to Miss Edith Wiegand for the development of the method for determination of cyprethylene ether in blood and to Miss Marjorie Ruppertsberger for blood chemistry determinations.

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# STUDIES ON PAIN: MEASUREMENT OF THE EFFECT OF ETHYL ALCOHOL ON THE PAIN THRESHOLD AND ON THE "ALARM" REACTION

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Ethyl alcohol has long been used by man for making pain and distress more bearable. It is probably the oldest analgesic agent. The purpose of this communication is to present quantitative measurements of the analgesic properties of this common agent in man, and briefly to evaluate its effect on the attitude and other reactions to pain.

**METHOD.** The method used for the quantitative measurement of the pain threshold raising action of 95 per cent ethyl alcohol was the same as has been used in the investigation of other analgesic agents reported elsewhere (1, 2, 3, 4). Heat from a 1000 Watt electric bulb was focussed by a lens through an aperture 3.5 cm.<sup>2</sup> in area onto the blackened forehead of the subject. The amount of heat just provoking a painful sensation was measured by a radiometer. This amount of heat stimulus was designated the pain threshold, and was expressed in gram calories per second, per square centimeter. Several control readings of this normal pain threshold were made at intervals of 5 to 10 minutes before the quantity of analgesic agent to be assayed was administered by mouth. Pain threshold readings were then made at ten minute intervals until the threshold had returned to the control level.

**Experimental subjects.** The subjects for the experiments were the three authors of this paper, two men and one woman. They were of approximately the same weight (65-66 kilos) and age (35-42 years), but of different personality types and body builds. All were accustomed to occasional alcohol consumption. Thus, neither ignorance of the effects of alcohol, nor tolerance to its use were factors to be considered. Furthermore, the three subjects were skilled observers, trained in manipulating the apparatus and in maintaining an objective attitude toward the experimental procedure.

**Procedure.** Three independent protocols were made, so that no subject could know until the end of the experiment how much his own threshold had been altered. As in previous experiments with other agents, concise statements of the psychological state were made and recorded at each ten minute interval when pain threshold readings were made. In the interims the subjects sat comfortably, engaged in reading, writing or conversation or, if drowsy they walked about the laboratory.

Experiments were begun from three to five hours after the last meal, and no food was eaten until after the peak of the threshold raising effect had been passed. Lunches consisted uniformly of bread and milk.

The pain threshold raising effect was expressed in per cent elevation of the threshold above the control level, or, in other words, the percentage increase in stimulus necessary just to evoke the sensation of pain. Time action curves were made by plotting the average per cent elevation of threshold for the three subjects (ordinate) with the time or duration of effect (abscissa). The time action curves for ethyl alcohol were analysed in three ways: 1) the maximum height of the pain threshold raising action; 2) the length of the period of effectiveness; and 3) the total threshold raising action of the agent.

Amounts of ethyl alcohol from 15 cc. to 90 cc. were diluted approximately 1 to 4 with a

sweetened carbonated water, and were imbibed within a three minute period. Also thirty cubic centimeters of 95% ethyl alcohol was administered at once and one half, and at one hour intervals, to a total of 90 cc. Further, the effect of experimentally induced pain on the threshold raising action of ethyl alcohol was observed, and the effect of alcohol on the threshold for the reaction to distress, called herein the threshold for the "alarm" reaction, was also studied. Finally, the threshold raising effect of a combination of 30 cc. of alcohol and 0.3 gm. of acetylsalicylic acid was assayed. There was a total of 63 series of observations.

**THE EFFECT OF ETHYL ALCOHOL ON THE PAIN THRESHOLD.** *Observations.* The height of the threshold raising effect of 95% ethyl alcohol increased with amount administered from 15 to 30 cc. Whether the amount administered was large

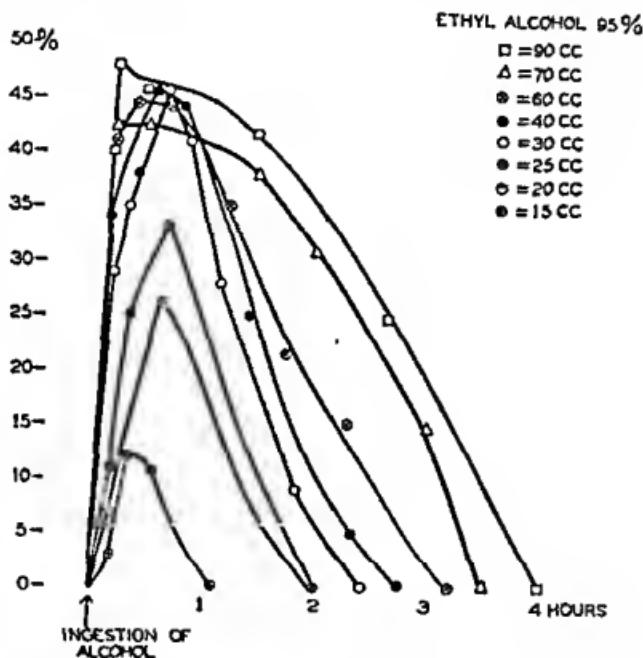


FIG. 1. TIME ACTION CURVES FOR 95 PER CENT ETHYL ALCOHOL

The ord' The absc' zero. The effect of the absorption of ethyl alcohol.

or small, the pain threshold was observed to rise within ten minutes, and the rate of rise was swift. The rate of rise also increased with amount given, the peak effect being reached within 40 minutes for the smaller amounts, and within 15 minutes for the larger amounts of 70 and 90 cc. The maximum elevation of the threshold was attained with 30 cc. of 95% ethyl alcohol, which was approximately 45% above the control level. It was not consistently elevated any further with larger amounts, i.e. 40, 50, 60, 70 and 90 cc. although, as already mentioned, the duration of effect was somewhat prolonged, the rate of rise was swifter, and the effects on mood and attitude toward pain were more intense and longer lasting with the larger amounts. Time action curves for all amounts assayed are shown in figure 1.

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## DURATION OF THRESHOLD RAISING EFFECT

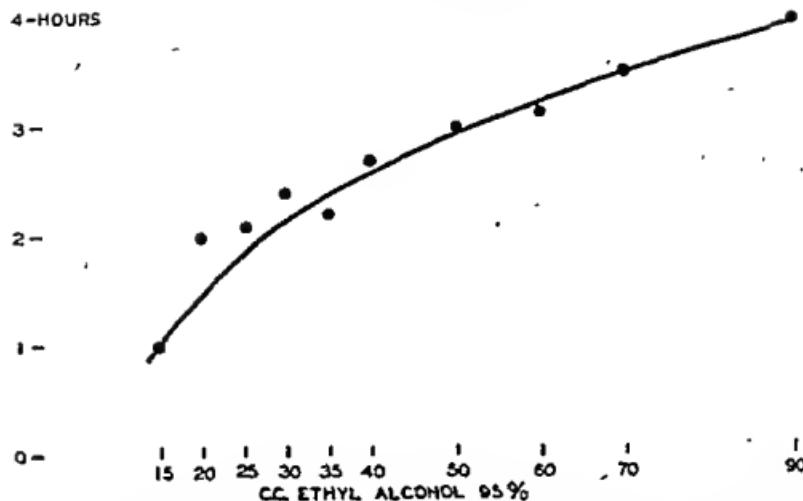


FIG. 3. THE RELATION BETWEEN DURATION OF THRESHOLD RAISING EFFECT (ORDINATE) AND THE QUANTITY OF 95 PER CENT ETHYL ALCOHOL ADMINISTERED (ABSCISSA)  
Each point represents the average for three subjects

## TOTAL THRESHOLD RAISING EFFECT

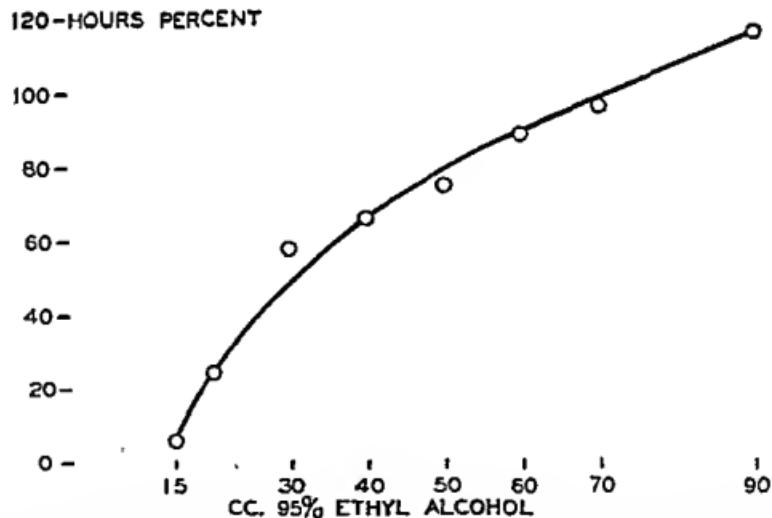


FIG. 4. THE RELATION BETWEEN THE TOTAL THRESHOLD RAISING EFFECT (ORDINATE) AND THE QUANTITY (ABSCISSA) OF 95 PER CENT ETHYL ALCOHOL ADMINISTERED

The ordinate was computed by multiplying the average per cent rise in pain threshold by the hours of duration of effect resulting from a given quantity of 95% alcohol.

cohol was administered in 30 cc. amounts at one and one-half hour, and in a second experiment at one hour intervals until a total of 90 cc. had been imbibed. Figure 5 shows that when given at one and one-half hour intervals the threshold

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## DURATION OF THRESHOLD RAISING EFFECT

4-HOURS

3 -

2 -

1 -

0 -

15 20 25 30 35 40 50 60 70 90  
CC. ETHYL ALCOHOL 95 %

FIG. 3. THE RELATION BETWEEN DURATION OF THRESHOLD RAISING EFFECT (ORDINATE) AND THE QUANTITY OF 95 PER CENT ETHYL ALCOHOL ADMINISTERED (ABSCISSA)  
Each point represents the average for three subjects

## TOTAL THRESHOLD RAISING EFFECT

120-HOURS PERCENT

100 -

80 -

60 -

40 -

20 -

0 -

15 30 40 50 60 70 90  
CC. 95% ETHYL ALCOHOL

FIG. 4. THE RELATION BETWEEN THE TOTAL THRESHOLD RAISING EFFECT (ORDINATE) AND THE QUANTITY (ABSCISSA) OF 95 PER CENT ETHYL ALCOHOL ADMINISTERED

The ordinate was computed by multiplying the average per cent rise in pain threshold by the hours of duration of effect resulting from a given quantity of 95% alcohol.

cohol was administered in 30 cc. amounts at one and one-half hour, and in a second experiment at one hour intervals until a total of 90 cc. had been imbibed. Figure 5 shows that when given at one and one-half hour intervals the threshold

raising effect began to subside considerably after each 30 cc. amount before the subsequent amount elevated the threshold again. However, when 30 cc. amounts were given at one hour intervals, a total of 90 cc. of alcohol maintained the pain threshold rather steadily at approximately 45% above its control level, for two and one-half hours. The total duration of effect of approximately 6 hours was noted in both instances (fig. 6).

*Comment.* In previous experiments with acetylsalicylic acid (4) it was observed that 0.3 gm. acetylsalicylic acid administered at 2 hour intervals was more effective than 0.6 gm. amounts at 3 hour intervals in maintaining the pain threshold at its maximum elevation. Thus, the total effectiveness of

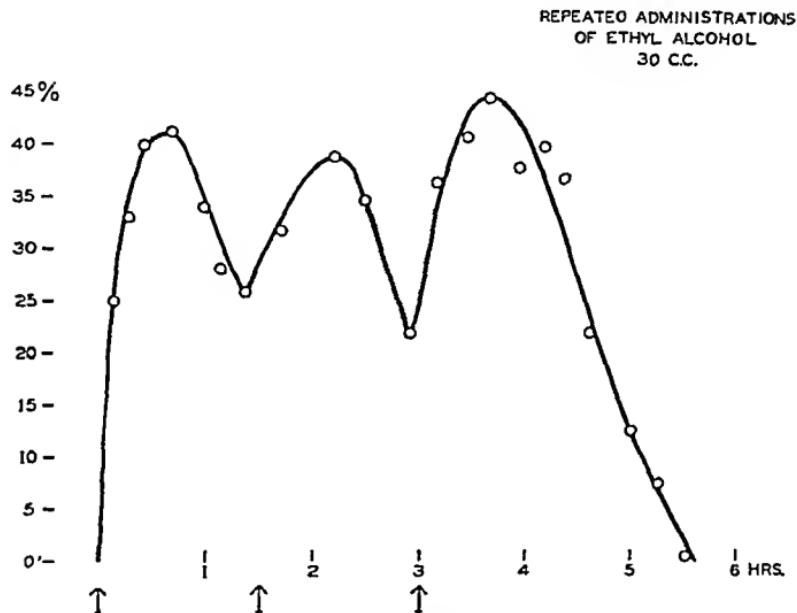


FIG. 5. THE EFFECT ON THE PAIN THRESHOLD OF REPEATED INGESTIONS OF 95 PER CENT ETHYL ALCOHOL, I.E., 30 C.C. AT ONE AND ONE-HALF HOUR INTERVALS

Each point represents the average of the threshold levels of three subjects. The arrows indicate the time of ingestion of the alcohol.

the agent was enhanced by administering it in smaller amounts at shorter intervals. Here, it was also observed that the duration of effect of a larger amount of ethyl alcohol, 90 cc., administered in 30 cc. amounts at hourly intervals was approximately two hours greater than the duration of pain threshold raising effect of an equal amount taken at one time. The threshold raising effect was maintained at or near its maximum elevation for a correspondingly longer time than with a single administration of this amount. Moreover, 30 cc. administered at hourly intervals was more effective than at one and one-half hour intervals.

THE EFFECT OF ETHYL ALCOHOL IN COMBINATION WITH ACETYLSALICYLIC ACID ON THE PAIN THRESHOLD. In figure 7 is shown a comparison of the threshold

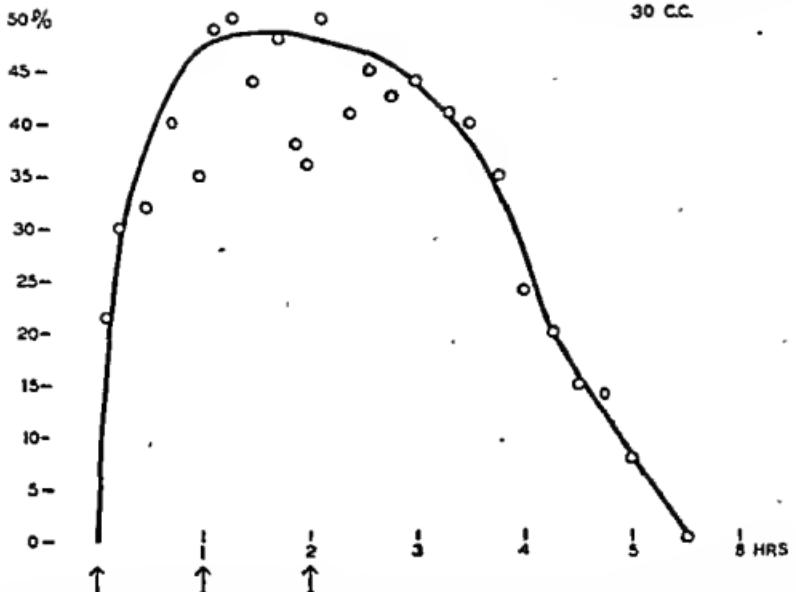


FIG. 6. THE EFFECT ON THE PAIN THRESHOLD OF REPEATED INGESTIONS OF 95 PER CENT ETHYL ALCOHOL, I.E. 30 CC., AT ONE HOUR INTERVALS

Each point represents the average of the threshold levels of three subjects. The arrows indicate the time of ingestion of the alcohol.

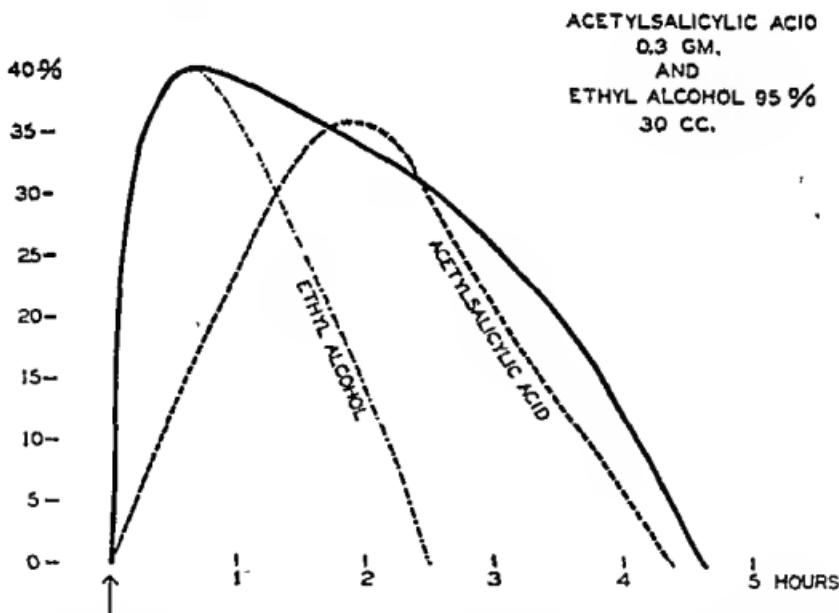


FIG. 7. THE EFFECT ON THE PAIN THRESHOLD OF A COMBINATION OF 95 PER CENT ETHYL ALCOHOL, 30 CC., AND ACETYLSALICYLIC ACID, 0.3 GM. (SOLID LINE)

The effect on the pain threshold of each of these agents separately is indicated by the broken lines as labeled.

raising effects of 30 cc. of ethyl alcohol alone, and of 0.3 gm. of acetylsalicylic acid alone. There is also shown the effect on the pain threshold of a combination of these two agents in the same amounts. With this combination the maximum effect of the alcohol, i.e. 45% elevation above the control level, was attained, together with the greater duration of effect of the acetylsalicylic acid, i.e., slightly longer than 4 hours. The more pronounced psychological effects of the alcohol, such as relaxation, euphoria, freedom from anxiety, and dissociation of perception of pain from the fight-flight-anxiety reaction pattern to pain, were coupled with the mild effect of relaxation and lethargy accompanying acetylsalicylic acid. Also, the threshold to pain was elevated at a much swifter rate with this combination than with acetylsalicylic acid alone. The maximum elevation was attained within 30 minutes of administration of the two agents. [It would seem desirable to make therapeutic use of such a combination.]

**THE EFFECT OF PAIN ON THE THRESHOLD RAISING ACTION OF ETHYL ALCOHOL.** An intense pain was experimentally induced by wrapping a sphygmomanometer cuff about the upper arm, inflating it to 200 mm. of mercury, and leaving it in place for 40 minutes. Moderate muscular movement accelerated the onset and intensity of the pain. Sixty cubic centimeters of 95% ethyl alcohol imbibed ten minutes before the end of this period of pain was effective in raising the pain threshold to its usual elevation, of approximately 45% above the control level (see fig. 8). Moreover, within three minutes the subjects' attitude toward this intense pain was altered from one of anxiety and distress, to one of detachment and freedom from anxiety.

*Comment.* In these experiments with alcohol it seemed as if the pain, though still perceived, had suddenly been freed of its implications of danger. As mentioned above, the usual fight-flight-anxiety reaction pattern of pain was altered to one of freedom from anxiety, indifference, apathy, or detachment. It was observed with the opiates that pain would greatly reduce their threshold raising effect, depending upon the intensity and duration of the pain when the opiate was administered. Thus, alcohol may be said to have an advantage over the opiates in this respect, since its threshold raising ability is not appreciably altered when it is administered in the presence of intense pain. It also like the opiates has desirable effects on the attitude, and other reactions to pain.

**THE EFFECT OF ETHYL ALCOHOL ON THE REACTION TO PAIN, OR "ALARM."** It has been found that the threshold for the perception of pain sensation is remarkably constant from day to day over a period of a year. Regardless of the mood of the individual, and attempts to increase fatigue and irritability by staying awake throughout 24 hours, this threshold did not vary more than  $\pm 12\%$  from the mean. Also, the pain threshold was of this same constancy in a group of 150 individuals of diverse personalities, age, sex and experience. However, among this group statements regarding their reactions to painful experience varied all the way from the stoical, of not feeling pain at all, to those of considering themselves extremely sensitive (3).

These facts all pointed to the inference that, whereas the threshold for the

perception of pain is as uniform among men and women as is the pulse rate, or the number of red and white blood cells, certainly reactions to pain and alarming experiences must vary greatly. These reactions are the most apparent, and perhaps the most important aspects of distressing experiences. It has already been observed (2) that when morphine sulfate, 0.015 gm. is administered during pain, the pain continues to be perceived, but the characteristic fight-flight anxiety reaction pattern of pain no longer obtains. In other words, perception has been dissociated from reaction.

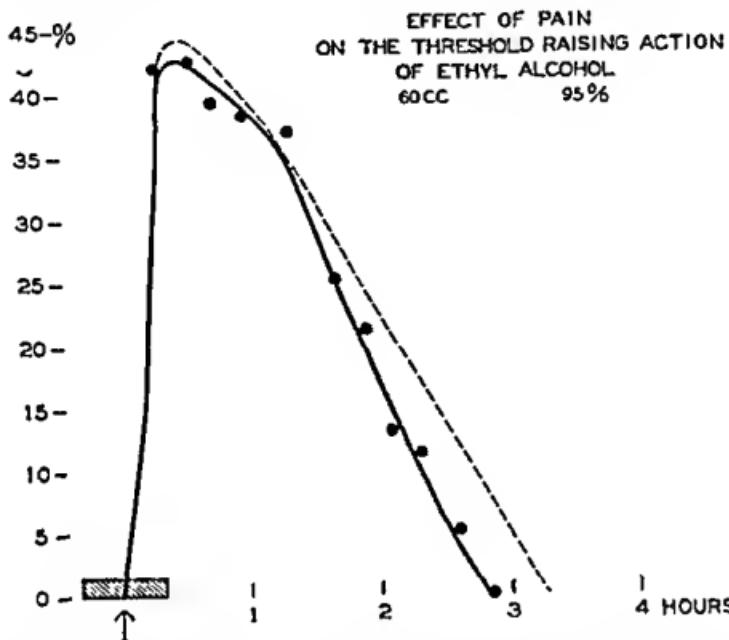


FIG. 8. THE EFFECT OF SUSTAINED PAIN FOR 40 MINUTES ON THE PAIN THRESHOLD RAISING PROPERTIES OF 95 PER CENT ETHYL ALCOHOL, 60 CC.

The broken line represents the average threshold raising effect of 60 cc. of 95% ethyl

of ingestion of the alcohol.

It was considered desirable to demonstrate this reaction to pain, as contrasted with pain perception, and to express some aspect of it in quantitative terms. One of the simplest components of the "alarm" reaction was used for this purpose, namely, the change in the electrical resistance of the skin resulting from the increase in sweat production occurring in moments of alarm or stress.

*Method.* The forearm and the middle finger were connected through electrodes in contact with the skin, to a Wheatstone bridge. The resistance between these points was measured and the galvanometer properly balanced. Thermal radiation was then applied for exactly three seconds to a 15 cm.<sup>2</sup> area of blackened forehead of the subject, and the

intensity of the radiation increased until the galvanometer needle swung sharply across the scale, following the stimulus. The amount of radiation just able to evoke this response to a heat stimulus on the forehead, was called the threshold of the "alarm" reaction. For comparison, and using the large area of  $15 \text{ cm.}^2$  the pain threshold was also determined in the manner described above.

*Observations.* The amount of heat necessary to evoke this "alarm" reaction was widely variable from day to day in the same individual, and from individual to individual in the three persons intensively studied. It was sometimes as little as  $0.027 \text{ gm. cals./sec./cm.}^2$  which was a fraction of the energy ( $0.21 \pm 0.02 \text{ gm. cals./sec./cm.}^2$ ) required to evoke the sensation of pain (see fig. 9). On

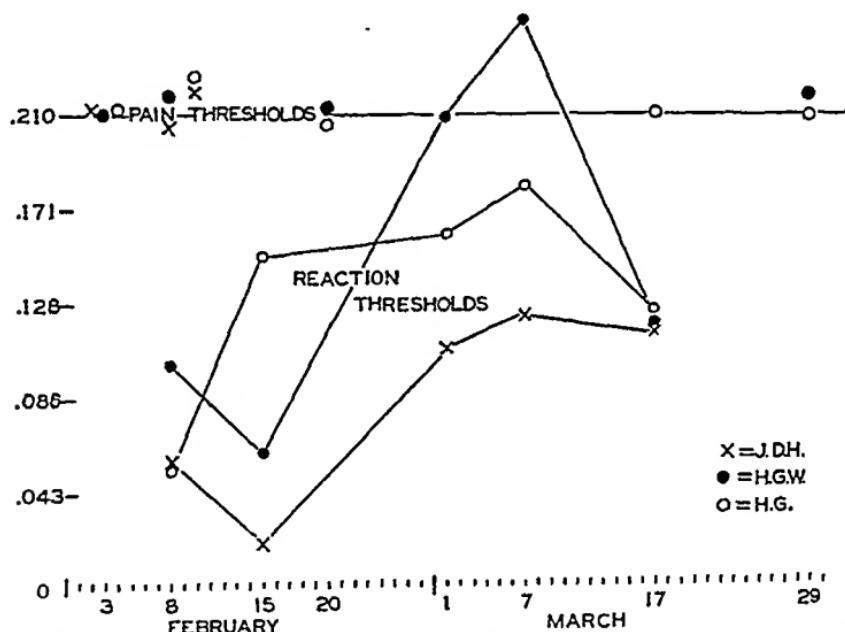


FIG. 9. A COMPARISON OF THE PAIN THRESHOLDS WITH THE "ALARM" REACTION THRESHOLD IN THREE SUBJECTS, UNDER CONTROL CONDITIONS

The ordinate = the intensity of stimulus, i.e., the amount of heat in gram calories per second per square centimeter. The abscissa = the date of the observations. Note the variability of the reaction threshold as compared with the stability of the pain threshold.

the other hand, when 40 cc. of 95 per cent ethyl alcohol was administered to the subjects of this study, their thresholds to the "alarm" reaction were observed to be elevated within 10 minutes after ingestion of the alcohol (fig. 10). In each of the three subjects the level of the maximum elevation of the threshold for the "alarm" reaction exceeded that of the maximum elevation of the threshold for pain. In other words, after alcohol more energy was required to evoke the "alarm" reaction, than to evoke the sensation of pain. In one instance the threshold of the "alarm" reaction was raised 780% above its own control threshold after 30 cc. of 95% ethyl alcohol. This elevation was 85% above the control level of the pain threshold. With this amount of heat blistering of the skin occurred (see fig. 11).

Furthermore, after the ingestion of ethyl alcohol the duration of the threshold raising effect for the "alarm" reaction was from one to two hours longer than the duration of the pain threshold raising effect.

*Comment.* Alcohol has an immediate and dramatic effect upon the threshold of the "alarm" reaction to a heat stimulus. The effect upon this threshold

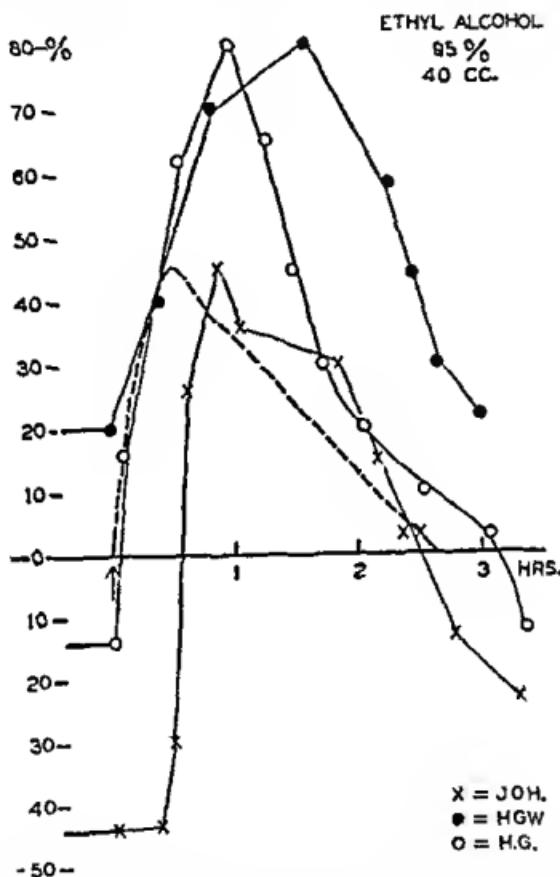


FIG. 10. THE EFFECT OF 95 PER CENT ALCOHOL, 40 CC., ON THE THRESHOLD OF THE "ALARM" REACTION

Each point represents the mean.

outlasts the effect on the pain threshold. Thus, there is afforded a demonstration of the distinction between the perception of pain, and the reaction pattern to pain. The "alarm" reaction to a heat stimulus appears to depend upon the emotional state of an individual, and upon his experience. Experience had conditioned these subjects so that heat became an unpleasant forerunner of burning pain.

Such a dissociation of pain perception from pain reaction, and the alteration of the "alarm" reaction is of importance in evaluating the therapeutic action of analgesics. Some agents, such as the salicylates, affect chiefly the pain threshold, while others, such as the opiates and alcohol, affect also the reaction pattern. Suggestion, reassurance, and confidence also influence the reaction

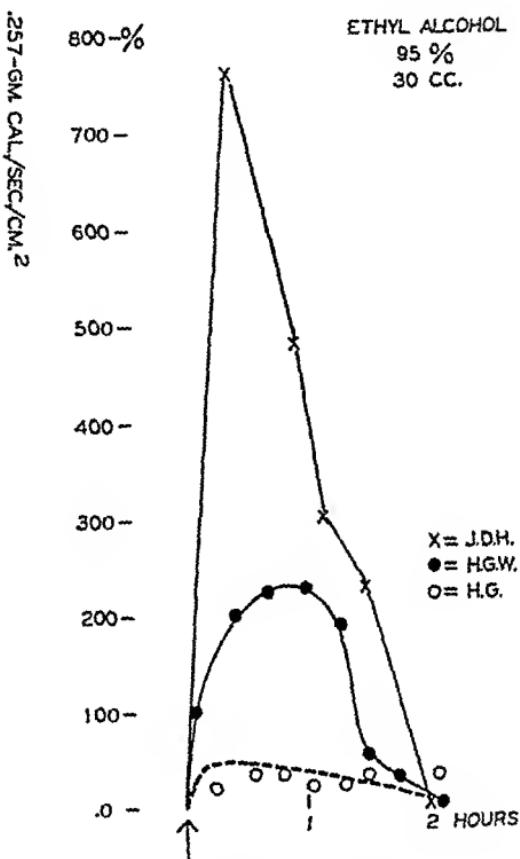


FIG. 11. THE EFFECT OF 95 PER CENT ETHYL ALCOHOL, 30 CC., ON THE THRESHOLD OF THE "ALARM" REACTION

The ordinate = the per cent elevation of the threshold of the "alarm" reaction above its control level as zero. The abscissa = the duration of effect.

pattern, and account for the enormous individual variations in the threshold for the "alarm" reaction.

It is to be noted that this "alarm" reaction commonly set off by pain, may and frequently does follow a completely innocuous stimulus. This would account for the observations frequently made on patients, namely that apparently non-painful stimuli cause the same reaction as do painful ones, that patients react differently to the same stimulus, painful or not, on different days, that a group of patients react variably to ostensibly the same stimulus.

## SUMMARY AND CONCLUSIONS

1. Quantitative measurements of the pain threshold were made by irradiating 3.5 em.<sup>2</sup> of skin surface for 3 seconds. The intensity of radiation which barely evoked pain was denoted as the pain threshold. The threshold raising action of ethyl alcohol was then ascertained in terms of the normal threshold.

2. Amounts of ethyl alcohol from 15 to 90 cc. were assayed. The smallest amount of ethyl alcohol with which the highest threshold raising effect was attained was 30 cc. The maximum pain threshold raising effect was approximately 45% elevation above the control level. The duration of effect was comparatively short, from one hour with 15 cc. to 4 hours with 90 cc. However, the rate of rise of the pain threshold after alcohol was swifter than with other agents which have been investigated, with the exception of inhaled trichlorethylene. The maximum elevation of the pain threshold after 90 cc. of alcohol was attained within 15 minutes, and after 30 cc. within 40 minutes.

3. A combination of 30 cc. of 95% ethyl alcohol with 0.3 gm. of acetylsalicylic acid had the advantages due to alcohol, namely, the swift elevation of the pain threshold, and the desirable feelings of contentment, relaxation and freedom from anxiety, coupled with the longer duration of the pain threshold raising effect of the acetylsalicylic acid.

4. When 95% ethyl alcohol was administered during a 40-minute period of experimentally induced pain, the pain threshold raising action of the alcohol was not significantly diminished. Moreover, the subjects continued to perceive the pain, but they were indifferent to it. Like the opiates, but perhaps to a lesser degree, alcohol accentuated the ability to dissociate pain perception from the fight-flight-anxiety reaction pattern of pain.

5. Change in skin resistance, one aspect of the commonly observed variation in reaction to distressing or painful experience, was measured. In contrast to the uniformity of the threshold for the perception of pain, the threshold for the "alarm" reaction varied widely from individual to individual and in the same person from day to day. The threshold for the "alarm" reaction was observed to be elevated up to 85% above the control pain threshold and as much as 780% above its own control level by 30 and 40 cc. amounts of ethyl alcohol.

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# THE EFFECT OF PAPAVERINE, EPINEPHRINE AND QUINIDINE ON THE FIBRILLATION THRESHOLD OF THE MAMMALIAN VENTRICLES<sup>1</sup>

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In previous papers, Wiggers and Wégria (1, 2, 3, 4) examined the physiological basis of a new method for determining the fibrillation threshold of the ventricles. Using the same technique, Wiggers, Wégria and Piñera studied the effect of procaine (4) and coronary occlusion (5) on the fibrillation threshold. Subsequently Wégria, Geyer and Brown (6) employed the method to study the effect of digitalis and ouabain. As reviewed in this communication, the method consists of applying a short D.C. stimulus of constant duration (e.g., 0.02 or 0.03 sec.) directly to the ventricle through non-polarizable electrodes in such a way that it falls during the vulnerable period of the heart cycle, i.e., late in systole or very early in diastole. The strength of such a shock (expressed in M.A.) which is just sufficient to cause the ventricles to fibrillate has been termed the fibrillation threshold shock. In order to study physiological, pharmacological or pathological factors which may influence the fibrillation threshold, it is necessary to repeat fibrillations before and after such influences. Consequently, in these experiments the fibrillation of the ventricles was promptly stopped by a short A. C. shock (or shocks) directly applied to the heart through two padded electrodes. As shown by Wiggers and Wégria (4), the fibrillation threshold repeatedly determined in this manner need not vary significantly over a 4-5 hour period in a dog under barbital anesthesia, provided certain experimental precautions are taken.

**METHODS.** Dogs averaging 10 kgm. in weight were used. The dog received intravenously 300 mgm. sodium barbital per kgm. of body weight. The chest was opened under artificial respiration through a midsternal incision and the heart suspended in a pericardial cradle. The mean arterial pressure was recorded with a mercury manometer connected with the left carotid artery. An electrocardiographic tracing, generally Lead III, was recorded from subcutaneous electrodes. The method of generating an electrical stimulus of desired strength and duration has been described previously (7). The sinus node was clamped and when the heart had assumed a slow nodal rhythm, it was driven at a constant rate by weak induction shocks applied on the right ventricle. Such a right ventricular pacemaker distributes impulses to the left ventricle via the left bundle branch as in supraventricular rhythms. It has the advantage over an atrial drive of the heart that A-V conduction disturbances introduced by some drugs are avoided. This is important in the use of this method, which requires an absolutely constant rhythm of the left ventricle in order to place shocks precisely in the vulnerable period (see previous communications).

**PAPAVERINE HYDROCHLORIDE. Results.** The value of papaverine in reducing liability to ventricular fibrillation was suggested by the work of McEachern,

<sup>1</sup> Supported by a grant from the John and Mary R. Markle Foundation.

Smith and Manning (8) who concluded that use of papaverine reduces the mortality of dogs following ligation of a coronary artery from 75 to 50 per cent. Lindner and Katz (9) reported that the use of this drug decreases the ease with which ventricular fibrillation is induced in the dog's heart by faradic stimulation; further, that the fibrillating ventricles can sometimes be revived by papaverine

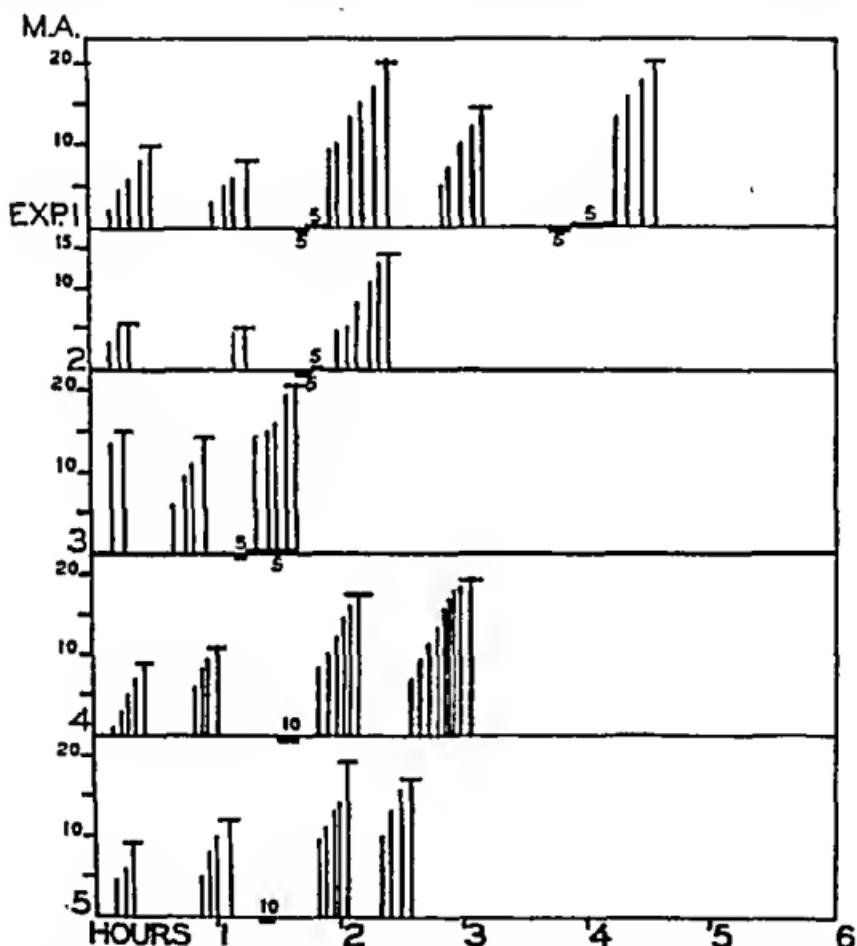


FIG. 1. PLOT SHOWING THE FIBRILLATION THRESHOLD OF THE DOG'S VENTRICLE BEFORE AND AFTER PAPAVERINE HYDROCHLORIDE

Each vertical line represents

papaverine.

used simultaneously with cardiac massage. Several reasons were previously given why we cannot regard such experiments as definitely conclusive. Therefore, we determined the changes in fibrillation threshold by our method before and after intravenous administration of papaverine. The results of five experiments are summarized in figure 1. In this, as in succeeding plots, each vertical

line represents the strength in milliamperes of a stimulus of constant duration, which, applied during a vulnerable period, produced or failed to produce fibrillation. The fibrillation current in each instance is designated "T."

In experiment 1, for instance, successive D.C. stimuli of 2, 4, 6 and 8 M.A. did not produce fibrillation, but a stimulus of 10 M.A. did so at 11:35 a.m. After prompt defibrillation and an equilibration period of 30 minutes, stimuli of 3, 5 and 6 M.A. did not cause fibrillation, but 8 M.A. produced fibrillation of the ventricles at 12:15 p.m. After revival and a recovery period of 20 minutes, a total dose of 10 mgm. of papaverine hydrochloride per kgm. was given via the femoral vein over a period of 15 minutes. At the end of the infusion successive stimuli of 9, 10, 13, 15 and 17 M.A. did not produce fibrillation and the strength of the stimulus had to be increased to 20 M.A. in order to induce ventricular fibrillation. After revival and the usual recovery, successive shocks of 5, 7, 10 and 12 M.A. did not cause fibrillation, but a shock of 14 M.A. did. After revival and recovery another dose of 10 mg. per kgm. of papaverine hydrochloride was administered, and this again increased the fibrillation threshold to 20 M.A. Experiments 2, 3, 4 and 5 show essentially similar results. Such doses of papaverine hydrochloride did not change the mean arterial pressure significantly in these experiments. In one experiment, not reported here, 10 mgm. of papaverine hydrochloride were administered intravenously over a period of five minutes, and a spontaneous fibrillation of a very coarse type developed. Incidentally, the observation was made that the fibrillation induced by electrical stimulation after use of papaverine was much coarser than in untreated dogs. However, spontaneous recovery with massage and without employment of electrical countershock was never observed.

The conclusion seems warranted that papaverine hydrochloride, in doses within therapeutic ranges, raises the threshold for induction of fibrillation. We have obtained no evidence that it may be useful in producing spontaneous recovery.

*Epinephrine.* It remains a moot question whether epinephrine increases or decreases the liability of the heart to fibrillate. Numerous observations have been reported concerning the development of ventricular tachycardia or fibrillation when epinephrine was administered to animals previously sensitized by chloroform, benzol or cyclopropane. As recently reviewed by Meek (10), such studies all lead to the conclusion that chloroform, benzol or cyclopropane, when used in suitable animals, are agents which alter ventricular sensitivity or irritability, whereas epinephrine constitutes the exciting agent. They offer no information as to how epinephrine *per se* affects ventricular sensitivity or irritability. This problem has been studied by administering epinephrine and using an electrical current as the exciting agent. Using such a procedure, Hoff and Nahum (11) reported that epinephrine increases the sensitivity of the cat's ventricle. Smith and Mulder (12), on the contrary, concluded that the cat's ventricle, made to fibrillate by means of a tetanizing current, recovers spontaneously within a shorter time and more frequently after injection of epinephrine. The value of results obtained by such procedures remains questionable for reasons previously stated (4). Obviously, however, differences of opinion on this important problem arise because different criteria were used. Some of the differences may also be due to the doses of epinephrine employed.

We therefore reinvestigated the problem by determining the fibrillation thresholds by our method. The results of typical experiments are shown in figure 2.

In experiment 1, successive stimuli of 4, 6, 8 and 11 M.A. did not produce fibrillation, but 13 M.A. induced fibrillation at 12:57 p.m. In the following threshold determination stimuli of 7 and 8 M.A. did not cause fibrillation of the ventricle, but 11 M.A. did at 1:30 p.m. During these two determinations, the mean carotid pressure was approximately 100 mm.Hg. Between 1:45 and 1:55 p.m., 0.5 mgm. epinephrine was given via the femoral

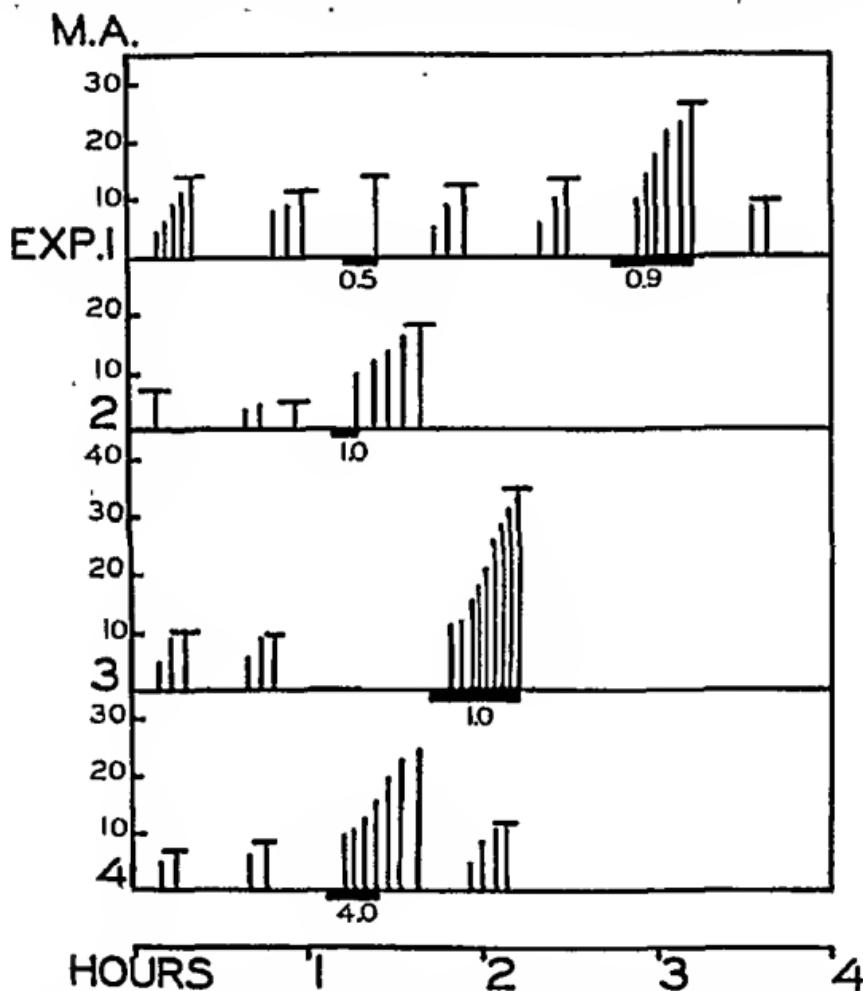


FIG. 2. PLOT SHOWING THE FIBRILLATION THRESHOLD OF THE DOO'S VENTRICLE BEFORE AND AFTER EPINEPHRINE

Arrangement same as figure 1. The numerals refer to the total dose of epinephrine in mgm. The heavy line under these numerals indicates the time and duration of the administration of epinephrine.

vein; at 1:55, a shock of 14 M.A. produced fibrillation and a weaker shock might possibly have done so. The threshold was acting, as was acting, as a recovery period.

shocks of 5 and 8 M.A. did not produce fibrillation, but 12 M.A. fibrillated at 2:25 p.m. After revival and recovery, while the mean arterial pressure was 100 mm.Hg., successive

shocks of 6 and 10 M.A. did not induce fibrillation, but 13 M.A. did at 3:05 p.m. The fibrillation was promptly stopped and a period of recovery allowed; then between 3:15 and 3:40 p.m., 0.9 mgm. epinephrine was administered. While arterial pressure was around 140 mm.Hg., successive stimuli of 10, 14, 17, 21 and 22 M.A. did not induce fibrillation, but 26 M.A. did at 3:40 p.m. The dog was revived and at 4:00 p.m. another threshold determination was begun. The threshold was 10 M.A., i.e., it was already back to its control value.

Experiments 2 and 4 show essentially similar results. In experiment 4 control fibrillation thresholds were 7 M.A. and 9 M.A.; immediately after epinephrine, fibrillation could not be induced even with stimuli as high as 25 M.A. Experiment 3 deserves a few additional comments. The first threshold determination was made while the mean carotid pressure was 150 mm.Hg. and the threshold was 11 M.A. During the second threshold determination, the pressure was about the same, 140 mm.Hg., and the threshold this time was 10 M.A. After revival the arterial pressure slowly decreased to 80 mm.Hg. One mgm. of epinephrine was then administered over a 30 minute period at such a rate that the arterial pressure was more or less kept constant around 150 mm.Hg. During the action of epinephrine, the threshold was tremendously increased; a stimulus of 35 M.A. was necessary to induce fibrillation, and that increase of threshold occurred despite the fact that the mean arterial pressure was the same as during the determination of both control thresholds. This evidently shows that, at least within certain limits, fibrillation threshold and mean arterial pressure do not undergo parallel changes.

We conclude that the fundamental effect of epinephrine is to cause an increase in the fibrillation threshold. In other words, it decreases the sensitivity of the heart to fibrillation by the electric current used in these experiments.

*Quinidine sulfate.* While the effects of quinidine have been repeatedly studied with respect to its value in preventing or abolishing ventricular tachycardia, it has not yet been demonstrated that such forms of tachycardia are necessarily related to induction of fibrillation. As regards direct experimental evidence, the opinions of investigators are divided. For example, Smith, McEachern and Hall (13) believe that it diminishes the risk of ventricular fibrillation following coronary occlusion, but Moisset de Epanès (14) was unable to obtain evidence of such preventative action. Such differing results are not surprising when the incidence of fibrillation following coronary occlusion is employed as a criterion. Although fibrillation may follow coronary occlusion, it depends on the fortuitous origin of properly timed stimuli. Ischemia itself causes a significant and variable decrease in threshold of sensitivity to electric shocks (Wiggers, Wégría and Piñera (5)). For these reasons, it seemed important to reinvestigate the effect of quinidine on the fibrillation threshold uncomplicated by such a factor as ischemia.

Results of typical experiments are shown in figure 3.

In experiment 1 of figure 3, short D.C. stimuli of 4 and 6 M.A. failed to cause fibrillation, but a 9 M.A. shock did so at 11:25 a.m. After prompt revival and an equilibration period of 20 minutes, another threshold determination showed that 6 and 8 M.A. failed, but 10 M.A. induced fibrillation at 11:55 a.m. After revival and equilibration for 30 minutes, 2 mgm. of quinidine sulfate per kilo were administered intravenously over a period of 20 minutes. This caused a temporary decline of mean carotid pressure of 20 mm.Hg., but the pressure returned to its control value before the end of quinidine administration and remained there during the fibrillation threshold determination. As shown in the chart of figure 3, successive stimuli of 6, 9, 11, 14 and 15.5 M.A. failed to cause fibrillation, but a

shock of 18 M.A. produced fibrillation at 1:10 p.m. Apparently a dose of quinidine sulfate (2 mgm./kgm.) administered intravenously which does not cause a permanent decrease of mean arterial pressure, reduces the sensitivity of the ventricle to fibrillation by an electric shock applied during the vulnerable period. Essentially similar results are shown in experi-

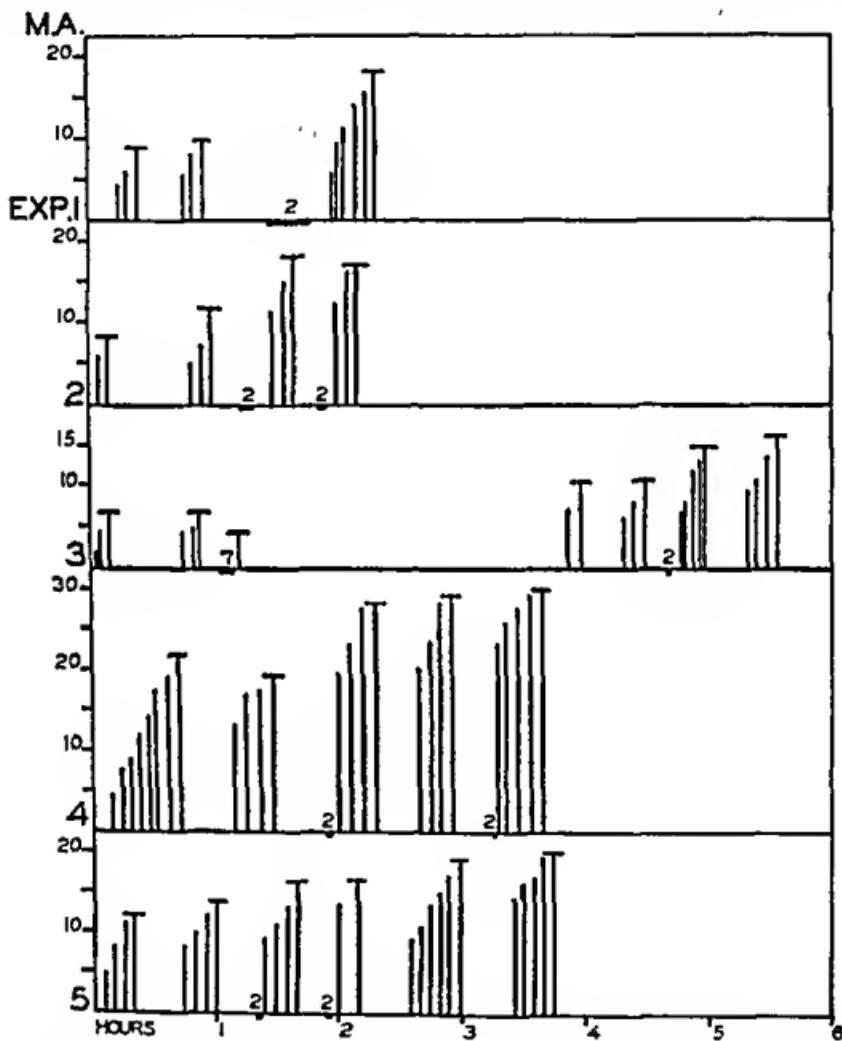


FIG. 3. PLOT SHOWING THE FIBRILLATION THRESHOLD OF THE DOO'S VENTRICLE BEFORE AND AFTER QUINIDINE SULFATE

Arrangement same as figure 1. The numerals refer to mgm. of quinidine sulfate per kilogram of body weight. The heavy line under these numerals indicates the time and duration of the administration of quinidine.

ments 2, 4 and 5 of figure 3. Experiment 3, however, demonstrates that, unless quinidine is properly administered, it may decrease the fibrillation threshold or leave it unchanged. As shown in the graph of experiment 3 in figure 3, short D.C. shocks of 2 and 4 M.A. failed to cause fibrillation, but a shock of 7 M.A. did so at 12:10 p.m. After prompt revival and following an equilibration period of 35 minutes, 4 and 5 M.A. did not initiate fibrillation

shocks of 6 and 10 M.A. did not induce fibrillation, but 13 M.A. did at 3:05 p.m. The fibrillation was promptly stopped and a period of recovery allowed; then between 3:15 and 3:40 p.m., 0.9 mgm. epinephrine was administered. While arterial pressure was around 140 mm.Hg., successive stimuli of 10, 14, 17, 21 and 22 M.A. did not induce fibrillation, but 26 M.A. did at 3:40 p.m. The dog was revived and at 4:00 p.m. another threshold determination was begun. The threshold was 10 M.A., i.e., it was already back to its control value.

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# INFLUENCE OF TEMPERATURE ON THE STIMULATION OF OXYGEN CONSUMPTION OF ISOLATED BRAIN AND KIDNEY BY 2-4 DINITROPHENOL<sup>1</sup>

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That the calorogenic action of 2-4 dinitrophenol (DNP) on intact animals is apparently lessened by exposure to cold has been reported by several investigators (1, 2, 3). An explanation of this effect is afforded by the work of Hall, Crisman and Chamberlin (4), who showed that administration of DNP to anesthetized cats depressed the cold defense mechanisms, as evidenced by decrease or abolition of shivering on exposure to cold. When allowance was made for this effect, it was shown that, within certain limits, the calorogenic action of DNP on the cat was enhanced by hypothermia. The intensity of the calorogenic action of the drug was greatest in the body temperature range 33° to 35°C. Continuing these studies, Hall and Chamberlin (5) showed that in anesthetized cats the increase in oxygen consumption evoked by DNP is markedly greater if epinephrine be infused intravenously at a physiological rate either just before or during the period of DNP action. Since in their earlier work a period of exposure to cold preceded administration of the drug, they inferred that such synergic calorogenic action is a factor in the increased metabolic stimulation caused by DNP in hypothermic animals.

The work reported here was undertaken to elucidate this phenomenon further. To this end we have investigated the percentage stimulation of respiration evoked by a given concentration of DNP in excised rat cerebral cortex and kidney cortex at temperatures ranging from 10° to 42°C.

Forty-one adult albino rats were used. These were killed by decapitation. Tissue slicing was performed in a moist box at 37°C. (6). Cerebral cortex slices were prepared as described previously (7); kidney cortex slices were made with a Terry microtome (8). All slices were weighed on a micro-torsion balance before immersion in Ringer's and then transferred to respirometer vessels. Oxygen consumption was measured by conventional manometric methods (9). The suspension medium was Ringer's glucose-phosphate; the gas phase, oxygen. Time between death of the animal and the beginning of measurement of respiration (cf. 10) was about 30 minutes. Each run lasted at least 90 minutes.

All measurements were made in triplicate and calculations were based on means so obtained. Readings were made at 10 minute intervals. DNP was added from the side-arms of the respirometer vessels 30 minutes after the run began. At this time control vessels received an equal volume of Ringer's. Control studies showed that under the conditions of these experiments, the respiratory rate of both tissues was constant for more than 2 hours.

All solutions were made up at pH 7.3, checked with a glass electrode. DNP was made up in Ringer's glucose-phosphate, and in all cases the final concentration of DNP corresponded

<sup>1</sup> Supported by grants from the Markle Foundation and from the Stanford University School of Medicine.

to 0.75 mgm. % of the sodium salt ( $3.35 \times 10^{-4}$  M). Subsequent work has shown that this is quite close to the optimum concentrations for stimulation of oxygen consumption in rat cerebral cortex at  $37.5^\circ$  and  $25^\circ\text{C}$ . ( $2.7 \times 10^{-4}$  M and  $1.8 \times 10^{-4}$  M respectively). It is also close to the concentration of 4:6 dinitro-o-creosol reported by Dodds and Greville (11) to be approximately optimum for the stimulation of rat cerebral cortex respiration at  $37^\circ\text{C}$ ., viz.  $1 \times 10^{-4}$  M.

Oxygen consumption is expressed in c.mm., N.P.T., per mgm. dry weight per hour ( $Q_{O_2}$ ). These figures were obtained by multiplying corresponding values of oxygen consumption per mgm. wet weight by the W/D ratios given in table 1.

**RESULTS.** 1. *Intensity of DNP action as a function of temperature.* A. *Cerebral cortex.* As Hall, Crismon and Chamberlin (4) have pointed out, the most satisfactory method of estimating the acceleration of oxygen consumption by DNP is by use of the stimulation ratio, i.e., the ratio of the oxygen consumption under the influence of the drug to that which would exist had the drug

TABLE 1

*Statistics: water content of cerebral cortex and kidney cortex in 20 adult male albino rats*

Tissues sliced in moist box. Wet weights made immediately on a micro-torsion balance without immersion in Ringers. Slices then transferred to crucibles and dried to constant weight at  $110^\circ\text{C}$ . in an electric oven.

	CEREBRAL CORTEX	KIDNEY CORTEX
Mean percentage water...	80.59%	75.66%
Range.....	79.0-82.7%	74.0-77.7%
Standard deviation.....	1.066%	1.508%
Standard error of mean.....	0.2384%	0.3371%
Coefficient of variation .....	1.323	1.9926
Mean W/D ratio*.....	5.15	4.11

\* W/D ratio =  $\frac{\text{wet weight}}{\text{dry weight}}$ ,  $Q_{O_2}$  on wet weight basis, multiplied by this factor, gives  $Q_{O_2}$  on dry weight basis.

not been administered. Expressing the former as  $Q_{O_2}$ ,DNP ( $Q_{O_2}$ , in the presence of DNP) and the latter as  $Q_{O_2}$ ,C ( $Q_{O_2}$ , in control vessels),

$$\text{Stimulation Ratio} = \frac{Q_{O_2},\text{DNP}}{Q_{O_2},\text{C}}$$

It is shown in table 2 that the intensity of the calorogenic action of DNP on cerebral cortex slices *in vitro*, as measured by the stimulation ratio, increased as the temperature rose from  $10^\circ$  to  $32^\circ\text{C}$ . (maximum), then decreased with further rise in temperature. The highest values of the stimulation ratio lay in the range  $23^\circ$  to  $36^\circ\text{C}$ . in which Hall and co-workers (4) observed active chemical defense against cold in the anesthetized cat. While the increment in  $Q_{O_2}$ , due to the presence of DNP (column III under "cerebral cortex," table 2) continued to rise with increase in temperature up to  $35^\circ\text{C}$ ., the greater rate of increase in  $Q_{O_2},\text{C}$  as compared with  $Q_{O_2},\text{DNP}$  resulted in a diminution of the stimulation ratio above  $32^\circ\text{C}$ . (table 2).

There is surprisingly good agreement between the temperature optimum for the stimulation ratio in excised rat cerebral cortex, 32°C. (table 2) and in the intact anesthetized cat, 33°C. (4). However, potentiation of DNP action by epinephrine, assumed by Hall and Chamberlin (5) to explain, in part at least, the rise in stimulation ratio in the hypothermic cat, is probably not a factor in the present experiments. The influence of temperature on the stimulation ratio observed in our work must be related to fundamental aspects of the metabolism of the brain itself or to exogenous factors which influence brain metabolism for several hours after excision or both.

If it be assumed that the influence of temperature on DNP action is substantially the same for cerebral cortex and for the hypothalamic temperature

TABLE 2

*Data showing the effect of  $3.55 \times 10^{-5} M$  DNP on the oxygen consumption of rat cerebral cortex and kidney cortex *in vitro*, at graded temperature levels*

Column I. Mean values of  $Q_{O_2}$  in vessels without DNP ( $Q_{O_2}C$ )  
 II. Mean values of  $Q_{O_2}$  in vessels with DNP ( $Q_{O_2}DNP$ )  
 III. Increment in  $Q_{O_2}$  due to presence of DNP ( $Q_{O_2}DNP - Q_{O_2}C$ )  
 IV. Stimulation ratio ( $Q_{O_2}DNP/Q_{O_2}C$ )

TEMPERATURE °C.	CEREBRAL CORTEX				KIDNEY CORTEX			
	I	II	III	IV	I	II	III	IV
10	1.34	1.91	0.57	1.42	1.81	2.06	0.24	1.14
15	2.63	3.97	1.34	1.51	2.92	4.11	1.19	1.41
18					3.53	5.10	1.57	1.44
20	3.40	5.67	2.27	1.67	4.48	6.78	2.30	1.51
22					4.93	7.52	2.59	1.52
25	4.07	7.21	3.14	1.77	6.70	8.92	2.22	1.33
28	6.03	10.81	4.78	1.79	7.23	9.58	2.35	1.32
32	6.75	12.72	5.97	1.88	9.66	12.04	2.38	1.25
35	8.70	14.83	6.13	1.70	14.30	16.81	2.51	1.18
37.5	10.40	15.40	5.00	1.48	15.91	15.54	-0.37	0.98
42	11.23	12.26	1.03	1.09	14.06	13.77	-0.29	0.98

centers, the present findings are in harmony with the hypothesis of Hall and co-workers (4) that abolition of the cold defense reactions by DNP may be due to the calorigenic action of the drug on the temperature centers, raising the threshold of these toward impulses from cold receptors or toward fall in blood temperature. Our finding that the calorigenic action of DNP on cortex slices *in vitro* is relatively greater over the temperature range in which the cold defense mechanisms are operative (in the cat at least) may be considered evidence in favor of this hypothesis.

*B. Kidney cortex.* To determine whether the findings just described were of more general application, it was necessary to carry out a similar investigation on tissues from another organ or organs. Kidney was selected because Clowes and Krahl (12) have shown that the percentage stimulation of oxygen consump-

tion of rat kidney by 4:6 dinitro-o-cresol and by certain halogenated phenols is greater at 20°C. than at 37°C.

It is shown in table 2 that the variation in stimulation ratio with temperature is quite different for the two organs. The maximum stimulation ratio (1.52) for kidney cortex occurred at 22°C., the maximum for cerebral cortex (1.88) at 32°C. This finding tends to emphasize the association of the maximum stimulation ratio in brain with the temperature range in which chemical defense against cold is most active (4). It may be noted that the concentration of DNP used,  $3.35 \times 10^{-4}$  M, actually caused slight inhibition of the respiration of kidney cortex at and above 37.5°C., whereas it evoked moderate increase of oxygen consumption by cerebral cortex up to 42°C. This disappearance of stimulation of kidney slice respiration by DNP at body temperature levels, using a concentration effective in other organs at this temperature, may account for the report by Dodds and Greville (11) that the respiration of kidney is not increased by DNP in Ringer's phosphate, since their work was done at 37°C.

*C. General discussion.* A satisfactory fundamental interpretation of these observations depends upon an understanding of the mechanism of action of the nitrated phenols in increasing the oxygen consumption of tissues. In spite of the accumulation of much relevant data (cf. Edsall, 13; Oppenheimer and Stern, 14), no certainty has yet been reached on this subject. Two major hypotheses have been offered in explanation.

- I. The nitrated phenols act as supplementary hydrogen transporters, through reversible oxidation and reduction (cf. 15).
- II. The nitrated phenols serve to increase the concentration of some substance or substances which can then be used as substrate by respiratory enzymes and related catalysts (14, 16).

Greville and Stern (15) have shown that the first hypothesis must be considered improbable. There is, however, considerable experimental support for the second. Thus it has been shown that in many instances the nitrated phenols evoke a marked increase in anaerobic sugar breakdown, and, in some tumors, an increased aerobic glycolysis (cf. 14). These observations suggest that the increase in oxygen consumption may be consequent upon increased substrate supply furnished by fermentative breakdown of sugar. Interesting and important in this connection is the observation of Clifton (17) that certain micro-organisms, which usually partly oxidize and partly synthesize a given substrate, oxidize the substrate completely in the presence of DNP and certain other cell poisons such as azide. If such a mechanism be operative in the mammalian cell, stimulation of respiration by the nitrated phenols is a secondary effect and the action of these drugs on enzyme systems may be purely inhibitory. On this view, the lower concentrations of DNP would inhibit the more sensitive synthetic enzyme systems, higher concentrations would inhibit both synthetic and respiratory enzymes. Quantitative differences in such synthetic reactions would then explain the quantitative variation in the effect of DNP on the oxygen consumption of brain and kidney, while the variation in stimulation ratio in a single organ

with change in temperature might result from a change in metabolic pattern with temperature.

2. *Temperature coefficient of respiration in cerebral and kidney cortex.* The ratio of the rate of a chemical reaction at a given temperature to the rate of that reaction at a temperature 10°C. lower is called  $Q_{10}$  (cf. 18). The present experiments provide data for the calculation of  $Q_{10}$  for oxygen consumption in cerebral and kidney cortex of the rat over a wide temperature range. For this purpose the values of  $Q_{10}C$  given in table 2 were plotted against temperature and fitted with smoothed curves.  $Q_{10}$  values were read from the smoothed curves at 5°C. intervals from 10° to 35°C. and at smaller intervals thereafter. The values of  $Q_{10}$  calculated from these data by the usual formula (18) are given in table 3.

It is shown in table 3 that the  $Q_{10}$  for respiration in rat cerebral and kidney cortex decreases with rise in temperature from 10° to 30°C. Such progressive diminution in  $Q_{10}$  with increase in temperature is characteristic of many biological

TABLE 3

$Q_{10}$  of the respiratory rate of cerebral cortex and kidney cortex at graded temperatures

TEMPERATURE RANGE °C	$Q_{10}$	
	Cerebral cortex	Kidney cortex
10 -15	2.07	2.52
15 -20	2.00	2.19
20 -25	1.83	2.01
25 -30	1.83	1.88
30 -35	2.11	2.75
35 -37.5	1.93	1.54
37.5-42	1.16	0.76

processes (18). However, both brain and kidney respiration show a definite rise in  $Q_{10}$  in the range 30° to 35°, followed by a decrease with further rise in temperature. A similar rise in  $Q_{10}$  as the temperature approaches the thermal optimum has been observed in certain crustacean tissue by Belding, Field, Weymouth and Allen (19), in sea urchin eggs by Korr (20) and in some other instances (cf. 18).

#### SUMMARY

The intensity of the acceleration of oxygen consumption of excised rat cerebral cortex and kidney cortex by a given concentration of DNP ( $3.35 \times 10^{-5}$  M) varies with temperature. The maximum percentage increase in respiration occurred at 32°C. in cerebral cortex and at 22°C. in kidney cortex. These observations support the hypothesis of Hall (4) that abolition of the cold defense reactions in hypothermic cats by DNP is consequent upon a relative increase in the metabolism of the temperature regulating centers with accompanying decrease in the sensitivity of these centers to the effects of cold stimuli.

Values of the temperature coefficient,  $Q_{10}$ , for the respiration of excised cerebral and kidney cortex were calculated for small intervals over the range 10° to 42°C. For both tissues there was a progressive decrease in  $Q_{10}$  from 10° to 30°C., followed by a definite rise in the range 30° to 35°C., with diminution on further rise in temperature.

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# STUDIES OF THE ADDICTION LIABILITY OF "DEMEROL" (D-140)

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This new drug,<sup>2</sup> originally described by Eisleb and Schaumann (1), was reported to possess definite analgetic and spasmolytic properties together with a mild atropine-like action. Subsequent reports confirming these findings were made by Duguid and Heathcote (2), and Schaumann (3). Early clinical reports confirming its analgetic and spasmolytic effectiveness were made by Dietrich (4), Shaefer (5), Rosenthal (6), Klein (7), and Althoff (8). Later, clinical reports suggesting that Demerol might be habit-forming were made by Von Brucke (9) and Kucher (10). The latter noted withdrawal symptoms when the medication was withheld and clonic muscular twitchings following its administration. In view of this, it seemed advisable to ascertain whether or not Demerol possesses the quality of satisfying or producing physical dependence.

**METHODS.** *Substitution.* Thirteen addicts with established physical dependence were stabilized on the respective minimal amounts of morphine needed to prevent signs of withdrawal. One week later Demerol was completely substituted for morphine. In each instance an attempt was made to duplicate the clinical picture obtained with morphine by adjustment of the amounts and intervals of administration of Demerol. The morphine stabilization doses ranged from 25 to 50 mgm. 4 times daily; the Demerol substitution doses from 60 to 120 mgm. 6 to 12 times daily. The mean values for dosage were: morphine, 36 mgm. 4 times daily; Demerol, 86 mgm. 9 times daily.

After 10 days the administration of Demerol was abruptly discontinued. Observations for signs of withdrawal were made three times daily throughout, and at more frequent intervals for the first 48 hours after withdrawal. The abstinence syndrome intensity was scored by the point system shown in table 1.

*Addiction.* Demerol was administered regularly to four prisoner patients (former addicts) selected from volunteers with sentences of sufficient length to permit full recovery prior to release. The medication was administered hypodermically in progressively increasing amounts at intervals consonant with the condition and desires of the subjects (table 2). The administration of Demerol was interrupted for one day after the first and after the second months of study, and was abruptly discontinued in the 10th or 11th week. Observations for signs of withdrawal were made and the abstinence syndrome was scored as described under Substitution.<sup>3</sup>

**RESULTS.** *Substitution.* Support of morphine physical dependence by Demerol was incomplete (fig. 1), but after its withdrawal signs of abstinence became intensified for two days, then waned. The results of studies of codeine by substitution (11), and the standard morphine abstinence syndrome (12), are shown (fig. 1) for comparison.

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<sup>2</sup> 1-methyl-4-phenyl-piperidine-4-carbonic acid ethyl ester, known as "Dolantin" in Europe, was furnished by Dr. O. W. Barlow of the Winthrop Chemical Company.

<sup>3</sup> Because of the presence of tremor during the administration of Demerol, this abstinence sign was not scored in the addiction studies.

TABLE 1

Point system for measuring abstinence syndrome intensity by the day (D) or by the hour (H)

SIGNS	(D) BY DAY		(H) BY HOUR	
	Points	Limit	Points	Limit
Yawning.....	1	1	1	1
Lacrimation.....	1	1	1	1
Rhinorrhea.....	1	1	1	1
Perspiration.....	1	1	1	1
Mydriasis.....	3	3	3	3
Tremor.....	3	3	3	3
Gooseflesh.....	3	3	3	3
Anorexia (40% decrease in caloric intake).....	3	3	3	3
Restlessness.....	5	5	5	5
Emesis (each spell).....	5		5	5
Fever (for each 0.1°C. rise over mean addiction level).....	1		1	10
Hyperpnoea (for each resp./min. rise over mean addiction level).....	1		1	10
Rise in A.M. Systolic B.P. (for each 2 mm. Hg over mean addiction level).....	1	15	1	10
Weight loss (A.M.) (for each 1 lb. from 1st day of addiction).....	1			

Total abstinence syndrome intensity per day or per hour is the sum of the points scored in the (D) or (H) columns respectively, with due attention to the limits.

TABLE 2

## Dosage

WEEK	SUBJECT 1		SUBJECT 2		SUBJECT 3		SUBJECT 4	
	Dose	No. of Injections						
1st	7.250	72	2.625	43	6.525	65	7.625	74
2nd	11.225	84	4.800	56	9.375	74	10.750	81
3rd	14.000	78	0.675*	9	10.725	71	11.855	76
4th	14.200	71	3.100	39	15.000	70	11.520	70
5th	13.600	62	4.725	50	10.800	52	10.710	61
6th	16.640	59	5.805	55	12.600	70	13.730	77
7th	18.500	74	7.775	64	14.000	75	15.590	82
8th	19.120	74	9.590	73	15.300	74	16.510	85
9th	24.300	90	11.300	83	20.000	91	18.495	99
10th	21.060	78	10.925	81	18.480	85	19.590	95
11th			0.650	4	3.710	18	8.840	52
Totals....	159.895	752	61.970	557	136.515	755	145.216	852
Mean dose.....	0.212		0.111		0.181		0.170	
Total no. hours on Demerol†...	1634		1505		1656		1703	
Mean hourly interval....	2.17		2.70		2.19		2.00	

\* Voluntarily discontinued drug for 7 days.

† Less time of withdrawals.

Not only was the Demerol abstinence syndrome less severe than that of morphine or codeine by objective criteria, but the subjective complaints were markedly reduced. Some of the patients remarked that the effect of the medica-

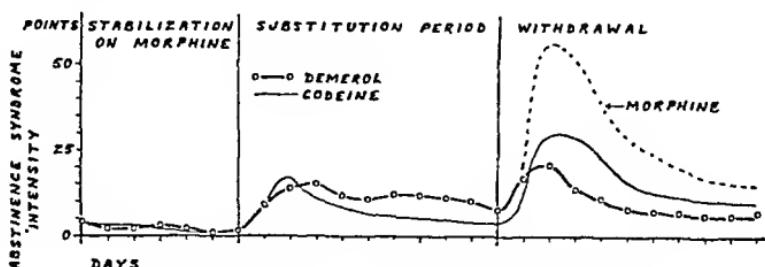


FIG. 1. SUBSTITUTION OF DEMEROL FOR MORPHINE

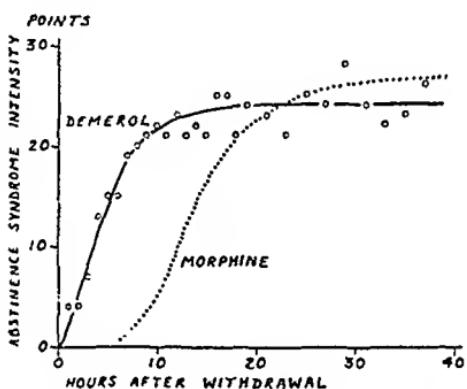


FIG. 2. ONSET OF DEMEROL ABSTINENCE SYNDROME

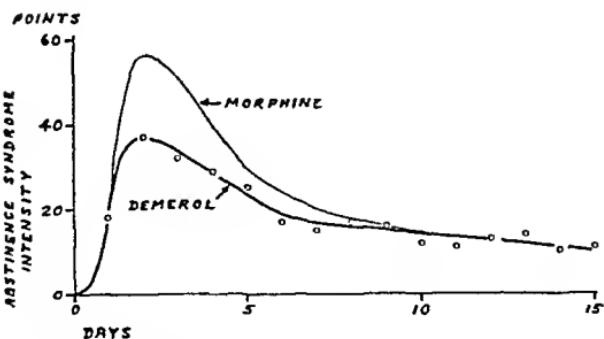


FIG. 3. WITHDRAWAL OF DEMEROL AFTER ADMINISTRATION FOR 10 WEEKS

tion was similar to atropine or hyoscine (one had a mild hyoscine-like delirium); the majority liked the effects and considered the substitution to constitute a "good treatment" for withdrawal.

The onset of the abstinence syndrome was more abrupt than that of morphine;

50% of the maximal intensity being reached in 4 to 5 hours, whereas this value for morphine is 14 hours (13).

*Addiction.* On withholding Demerol for 22 hours after one month of administration, definite but very mild signs of abstinence appeared by the 10th hour and persisted until administration of the drug was resumed. Nevertheless the patients complained of no symptoms and did not consider the drug to be "habit-forming." On withholding Demerol for 24 hours after two months of administration, definite but mild signs of abstinence appeared somewhat earlier; but again the patients had no appreciable subjective discomfort. Following withdrawal of the drug in the 10th or 11th week of study a definite abstinence syndrome appeared within a few hours and reached 50% of its maximal intensity by the 4th hour (fig. 2). The patients experienced none of the symptoms usually associated with withdrawal until the second day, when their complaints became typical of, although less severe than, those of morphine withdrawal. The objective criteria of abstinence likewise were less severe than, but otherwise quite typical of, the morphine abstinence syndrome (fig. 3).

During the course of the study the patients' temperatures, pulse rates, and systolic blood pressures became slightly elevated; whereas their respiration rates, appetites, and weights decreased somewhat. After the first week of administration the patients developed muscular tremors and twitches which continued throughout the course of study until the third or fourth day after withdrawal. Their reflexes were somewhat hyperactive and they seemed to become startled by stimuli ordinarily non-disturbing. One developed a mild toxic psychosis which cleared up on reducing the dose. Two of the patients had epileptiform (*petit mal*) seizures during the 10th week.

All the patients considered the effects of the drug to be nearly like morphine, but less well sustained, and all complained of dryness of the mouth throughout.

#### SUMMARY

When substituted for morphine Demerol partially satisfied the physical dependence established to morphine. On withdrawal, after 10 days of substitution, a definite but mild abstinence syndrome occurred.

Physical dependence on Demerol resulted from its regular administration to post-addicts over a period of 10 weeks. The abstinence syndrome which occurred following its withdrawal was milder than the morphine abstinence syndrome but otherwise quite typical. The duration of the physical dependence action of Demerol was considerably shorter than that of morphine.

#### CONCLUSION

Demerol possesses addiction liability.

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# THE ACTION OF SENECIONINE, INTEGERRIMINE, JACOBINE, LONGILOBINE, AND SPARTIOIDINE, ESPECIALLY ON THE LIVER<sup>1</sup>

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Since the isolation of retrorsine by Manske (2), there has been a renewal of interest in *Senecio* species, both chemically and pharmacologically. In addition to Manske's subsequent contributions (3, 4, 5), Barger, Seshadri, Watt, and Yahuta (6), Barger and Blackie (7), and Blackie (8) investigated the properties and the chemistry of several new alkaloids. Similarly, Orechoff (9), Orechoff and Konowalowa (10), and Konowalowa and Orechoff (11, 12, 13) described the chemical nature of platiphylline and seneciphylline. Recently, deWaal (14, 15, 16) studied the constitution of isatidine and other related alkaloids. In this laboratory, pharmacologic and toxicologic experiments have been carried out by Chen, Chen, and Rose (17) on retrorsine, and by Chen, Harris, and Rose (18) on platiphylline and seneciphylline. A pathological report on retrorsine in rats was also made by Davidson (19). It is interesting that lasiocarpine, an alkaloid of *Heliotropium lasiocarpum*, produces similar hepatic lesions as most alkaloids of *Senecio* species (20). The present report deals with the effects of integrerrimine, jacobine, senecionine, longilobine, and spartiodine in various animals. These alkaloids were isolated by Manske from *Senecio integrerrimus*, *S. jacobaea*, *S. longilobus*, and *S. spartoides*, respectively. The authors are indebted to Dr. Richard H. F. Manske, Division of Chemistry, National Research Council, Ottawa, Canada, for a supply of each specimen. Solutions were easily prepared by dissolving the weighed quantity of each alkaloid in an equimolecular amount of hydrochloric acid.

*Toxicity in mice.* On intravenous injection in mice, similar toxic effects were observed with all the 5 alkaloids. Large doses caused rapid respiration, clonic convulsions, and death within a few minutes. Animals receiving smaller doses might temporarily recover from convulsions, but they became lethargic, lost appetite, exhibited erection of hair, and died in 24-96 hours. If they survived 4 days after medication they usually began to eat and move around and soon appeared normal. Arbitrarily, 1 week was allowed before the experiment was concluded. The median lethal doses ( $LD_{50} \pm$  standard error) of senecionine, integrerrimine, jacobine, longilobine, and spartiodine were determined, as shown in table 1, on the basis of 7 days' observations. It is obvious that senecionine is the most toxic of the entire group, while the remaining 4 compounds are fairly close in their toxicity.

*Pathological studies.* Necropsies were carefully made on those mice which

<sup>1</sup> Read at the Chicago Meeting of the American Society for Pharmacology and Experimental Therapeutics on April 19, 1941 (1).

died, and also on those animals which survived a week, following single injections of these alkaloids as given in table 1. Chloroform vapor was used to kill the latter group immediately before postmortem examination. In the earlier experiments sections of the liver, lungs, kidneys, spleen, and adrenals were made routinely. Subsequently only the livers were sectioned routinely, but in the great majority of animals sections of the kidneys were also made.

As soon as it was revealed that the liver uniformly underwent necrosis additional experiments were carried out in mice by subcutaneous, oral, or intravenous administration of sublethal doses of the alkaloids. These doses were repeated frequently with the purpose of introducing a slow development of the lesion, so that it might resemble cirrhosis of the liver as it occurs in cattle after the ingestion of *Senecio* plants. The results may be summarized as follows.

1. *Senecionine*. Thirty-four mice which died after intravenous injection of single doses of senecionine were autopsied (table 1). Slight ascites occurred in 8 animals, moderate ascites in 2; slight hydrothorax in 9, moderate hydrothorax in 1; and pulmonary edema in 3.

Hepatic changes consisting of necrosis of liver cells, sinusoidal congestion, and hemorrhage into the cords of liver cells, took place in all animals. Necrosis on one hand, and congestion and hemorrhage on the other, were almost invariably associated, although sections of the livers of 2 mice showed no congestion, and sections of 3 others, intense sinusoidal congestion and hemorrhage with no evidence of necrosis. In 6 animals, necrosis was periportal; in 24, it was central; and in the liver of 1 mouse there was slight midzonal necrosis. In some cases hemorrhage into cell cords was so extensive that liver cells appeared to have been washed away. A similar appearance was mentioned by Davidson (19) with retrorsino in rats. This type of lesion was also produced by the 4 other alkaloids of the present series. In a little more than half the livers, focal accumulations of blood caused a resemblance to a cavernous hemangioma as illustrated in figure 1. The same lesion was referred to by Davidson (19) as the formation of "blood lagoons" or "blood pools." In places this angiomatoid appearance evidently resulted from hemorrhage into the liver cords with displacement of liver cells and compression of the sinusoids, but in other instances it could have resulted from sinusoidal distension. The lesion, severe and widespread in half the mice, was usually central in location, but in a few animals it was periportal, and in some cases the exact location was uncertain owing to its great extent and severity. In general, it was found in the region where necrosis was commonly observed. This also applies to the other 4 alkaloids of the present investigation.

As was to be expected, there was no constant correlation between the size of the dose and the survival time, and the type of lesion produced. Practically all animals receiving large doses died early, but a few mice on small doses also succumbed before the end of 24-48 hours. Usually moderate or extensive necrosis occurred with late death, but it was also present in the livers of a few mice which died early. Extreme congestion and hemorrhage into cell cords with or without evident necrosis were appreciably more common in animals which died early, although they were by no means confined to them. These statements also apply to jacobine, longilobine, integerrimine, and spartoidine, so that subsequent repetitions will not be necessary.

Eighteen mice surviving 7 days after the intravenous injection were chloroformed (table 1). The organs of 13 animals were normal; the livers of 2 on the

TABLE I  
*Toxicity of senecionine, integerrimine, jacobine, longilobine, and spartiodine*

ALKALOID	DOSE	NUMBER DIED NUMBER USED	LD <sub>50</sub> ± S. E.	NUMBER OF MICE AUTOPSIED	
				After death	After sacrifice
Senecionine.....	40	0/2	64.12 ± 2.24		
	56	3/10		3	7
	62	3/10		3	7
	70	6/10		6	4
	80	12/12		11	
	90	5/5		5	
	100	7/7		6	
	120	2/2			
Jacobine.....	40	0/2	77.11 ± 2.86		
	70	1/10		1	9
	80	10/12		10	2
	90	8/10		8	2
	100	9/10		9	1
	120	2/2		2	
	160	2/2		1	
Longilobine.....	40	0/2	77.85 ± 3.33		
	56	0/5			5
	62	0/10			10
	70	5/10		4	5
	80	8/12		6	4
	90	6/10		6	4
	100	5/5		5	
	120	2/2		1	
Integerrimine .....	40	0/2	78.32 ± 3.05		
	56	0/5			
	62	0/10			10
	70	4/10		4	6
	80	7/12		6	5
	90	3/5		2	2
	100	2/2			
Spartiodine. ....	120	2/2			
	40	0/2	80.39 ± 1.93		
	70	1/10		1	9
	80	5/12		5	7
	90	9/10		9	1
	100	10/10		8	2
	120	2/2		2	
	160	2/2		1	

dose of 62 mgm. per kgm. contained small groups of calcified cells; and the pancreas of a third showed edema and regions of necrosis. In 2 other mice receiving a dose of 70 mgm. per kgm., the cells in the center of some liver lobules were lost and replaced by monocytes.

2. *Jacobine*. Necropsies were made on 31 mice which died after a single intravenous injection of jacobine (table 1). Ascites was present in 15 animals—slight in 11, moderate in 3, and copious in 1. Hydrothorax occurred in 4 mice;

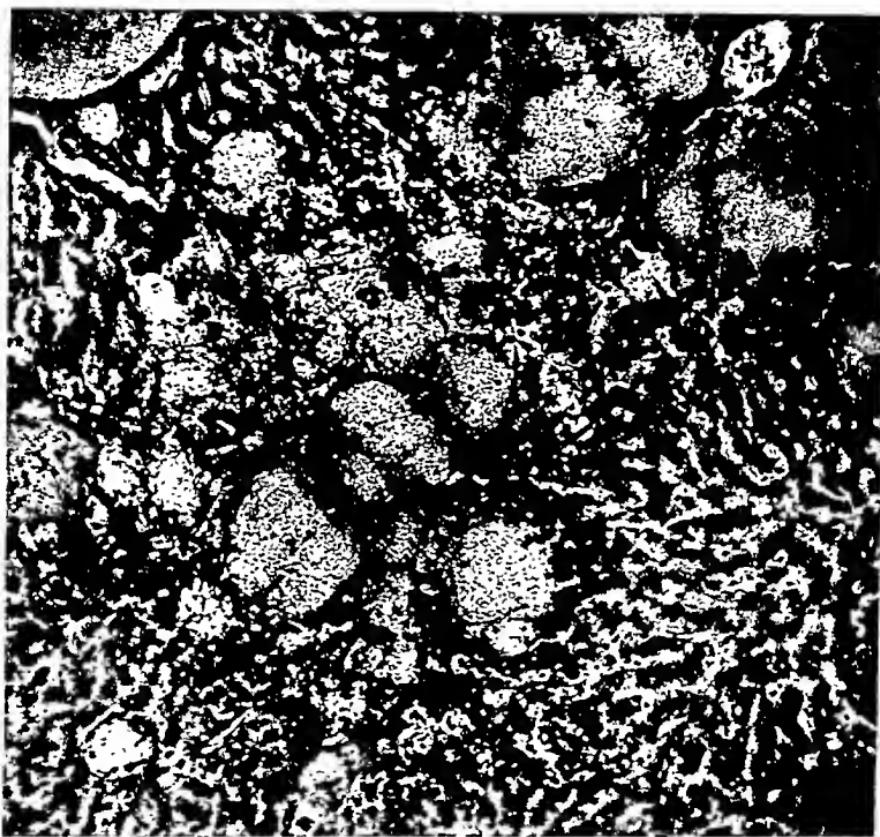


FIG. 1. ANGIOMATOID LESION PRODUCED BY SENEACIONINE.  $\times 162$

Mouse, numbered 229, male, weighing 17.5 gm., died 2 days after intravenous injection of scnecionine in the dose of 62 mgm. per kgm. This lesion involves the tissue in the center of the liver lobules. The black granules are due to the action of formalin upon hemoglobin.

and pulmonary edema, in 4. Microscopically, the livers of 4 animals were slightly congested, but all others showed much congestion with hemorrhage into cell cords. Production of an angiomatoid appearance was present in 14. Necrosis of the liver was obvious in 30 mice—23 central, 4 midzonal, and 3 periportal.

Of the 16 surviving animals (table 1), 14 were normal. The livers of 2 mice on the dose of 70 mgm. per kgm. contained groups of calcified cells, more in one than in the other. It is not certain that the occurrence of such cells may be due to jacobine.

A group of 13 mice was given subcutaneous injections of jacobine daily except Saturdays and Sundays. Five of them had 75 mgm. per kgm. each day, but died following the second dose. The lesions observed were the same as those after intravenous injection of single, large doses. The remaining animals, 8 in all, received repeated doses of 40 mgm. per kgm., and died in 26-70 days. Six had ascites; 5, pulmonary edema; and 3, hydrothorax. The livers of all 8, instead of necrosis, showed hypertrophy of cells. An example is given in figure 2. In several cases the hypertrophy was most pronounced about the portal spaces. With the exception of 1 liver, there was a corresponding enlargement of nuclei.

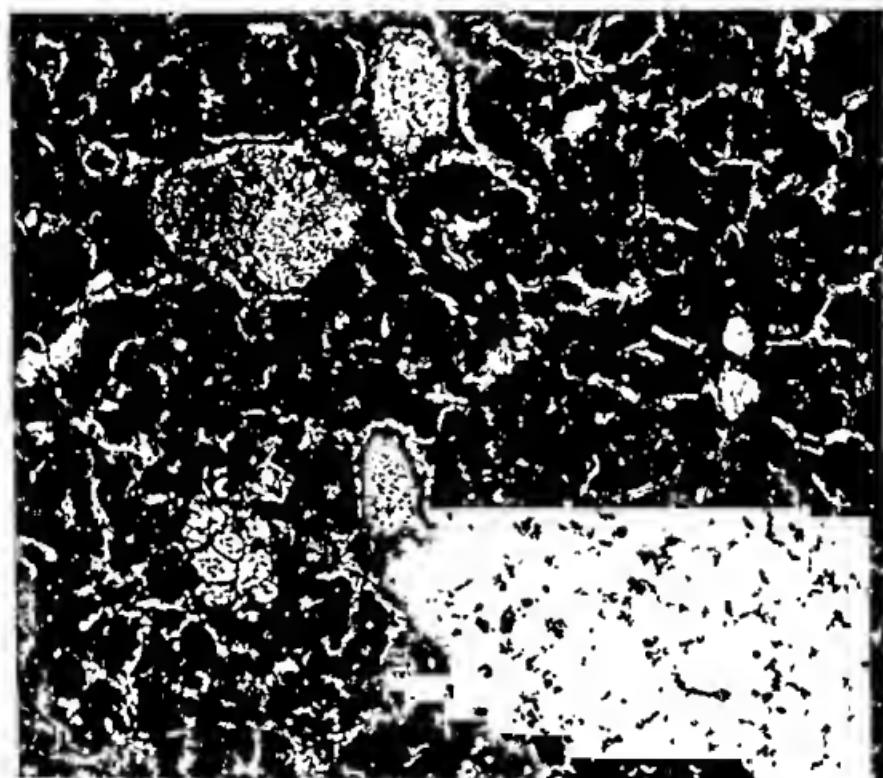


FIG. 2. HYPERSTROPHY OF LIVER CELLS BY PROLONGED ADMINISTRATION OF JACOBINE.  
X 162

Mouse, numbered 384, female, weighing 16 gm., received subcutaneously jacobine in the dose of 40 mgm. per kgm. five times weekly. It died in 39 days after 27 doses. The great increase in size of some cells and their nuclei is clearly evident. A small group of highly vacuolated cells is present.

Another group of 5 mice each received intravenously 60 mgm. per kgm. three times a week. One animal died in 2 weeks with hypertrophy of liver cells, another in 4 weeks with no evidence of liver pathology. After 4 weeks of intravenous injection the medication was continued in 3 remaining mice by subcutaneous injection in the same dosage. They were then sacrificed at the end of the ninth week counting from the first dose by vein. Two animals had ascites, and one had hydrothorax. Their livers showed hypertrophy of cells and nuclei.

A third group of 15 mice was given jacobine orally. The doses were 66, 280, and 569, mgm. per kgm., 5 animals being employed for each dose. Death resulted from the larger doses within 24 hours, and necropsies made on 8 mice disclosed the usual central hepatic

necrosis and congestion with hemorrhage into cell cords. The small dose, namely 66 mgm. per kgm., was repeated daily until death occurred. Those animals which died within 7 days also showed central necrosis, congestion, and hemorrhage. Only 1 mouse survived 20 days on that daily dose, and its liver cells were hypertrophic with neither necrosis nor fibrosis.

*3. Longilobine.* Postmortem examination was performed on 22 mice which died after a single intravenous injection of longilobine (table 1). Ascites was present in 12 animals; hydrothorax, in 10; and pulmonary edema, in 2. All animals showed necrosis of the liver—central in 19, periportal in 2, and diffuse,

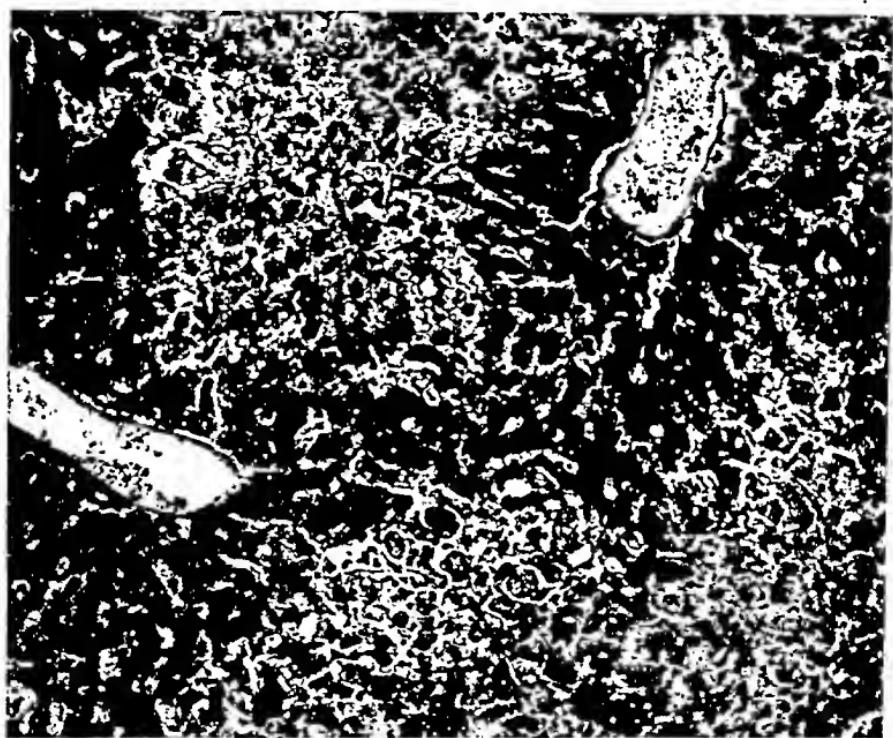


FIG. 3. CENTRAL NECROSIS CAUSED BY LONGILOBINE.  $\times 162$

Mouse, numbered 251, male, weighing 17.9 gm., died 3 days after intravenous injection of longilobine in the dose of 80 mgm. per kgm. There is extensive central necrosis of the liver with little leukocytic reaction.

in 1. Figure 3 is a good representative of the central necrosis. Congestion with hemorrhage also occurred. In the livers of 10 mice, an angiomatoid appearance was produced, particularly striking in 5.

Twenty-eight surviving mice were chloroformed at the end of 7 days following the intravenous injection (table 1). No abnormality could be demonstrated in 26 animals. In 1 mouse injected with 62, and in another injected with 80 mgm. per kgm., there was loss of cells in the center of a few liver lobules with replacement by monoeytes.

An additional series of 4 mice was given 40 mgm. per kgm. subcutaneously, every day except Saturdays and Sundays. They all succumbed within 8 weeks. The total quantity

of longilobine administered to each animal amounted to 800-1520 mgm. per kgm. Ascites was observed in 3 mice, and hydrothorax in one. The livers of all 4 showed hypertrophy of cells, and with the exception of one, hypertrophy of nuclei. In 2 mice the hypertrophic cells were seen only in the periportal regions.

4. *Integerrimine*. Twelve mice that died after the intravenous injection of single doses of this alkaloid (table 1) were studied pathologically. Ascites was found in 7 animals; hydrothorax, in 3; and pulmonary edema, in 2. The livers of all animals showed central necrosis accompanied by sinusoidal congestion and hemorrhage into cords of necrotic cells. An angiomaoid appearance was observed in 9 mice—especially striking in 5. Twenty-eight surviving mice were sacrificed with chloroform. Only 1 showed loss of cells in the center of liver lobules, and replacement by monocytes. The remaining 27 mice were normal.

An additional group of 5 mice received subcutaneous injections of 40 mgm. per kgm., daily from Monday through Friday each week. They died after the ninth, fifteenth, twentieth, twenty-first, and fortieth doses, respectively. All the livers showed evidence of hypertrophy, but no suggestion of cirrhosis.

5. *Spartioidine*. Of the 26 mice which succumbed to single intravenous doses of spartioidine, 8 had ascites, 4 hydrothorax, and 5 pulmonary edema. There was a definite sign of necrosis in the livers of 23 animals—18 central and 5 periportal. Sinusoidal congestion and hemorrhage into cell cords also occurred, angiomaoid in 8 cases. Of the 21 surviving mice, 20 were normal while one exhibited replacement of liver cells by monocytes in the center of some liver lobules.

A separate group of 17 mice was given intravenous injections of spartioidine daily. The doses varied from 10-20 mgm. per kgm. Thirteen animals were chloroformed after 9-16 doses, and were found to be normal. Four mice died during the intervals of 7-17 days, and their viscera were free from pathological changes. Only 1 animal, which died after 17 days, showed marked hypertrophy of liver cells in periportal areas.

Another group of 8 mice was injected intravenously once a week. The dose was the same for all of them, 60 mgm. per kgm. Five died in 23-89 days, but no lesions were present except slight focal calcification of liver cells in 1 animal. The remaining 3 were chloroformed, and were found to be normal.

A third group of mice received subcutaneous injections of spartioidine, in the dose of 40 mgm. per kgm. daily except Saturdays and Sundays. All died after a total amount of from 880-1600 mgm. per kgm. per capita had been administered. Hypertrophy of liver cells and nuclei uniformly occurred in their livers, particularly pronounced about the portal spaces.

*Other effects.* In etherized cats weighing 2-2.5 kgm., senecionine, integerrimine, jacobine, longilobine, or spartioidine, when injected intravenously in the dose of 30-50 mgm. (total), lowered carotid blood pressure usually with prompt recovery. All 5 alkaloids inhibited the peristaltic movements of isolated rabbits' intestines, in the concentrations of 1:50,000 and 1:40,000. Integerrimine, longilobine, or jacobine stimulated isolated guinea pigs' uteri in concentrations of 1:50,000 and 1:10,000, but both senecionine and spartioidine were ineffective.

in the solution of 1:10,000. In general their action on circulation and smooth muscle organs was similar to that of retrorsine, seneciphylline, and lasiocarpine (17, 18, 20).

DISCUSSION. It is apparent that all the 5 alkaloids have a similar toxic action in mice in that they all produce necrosis of the liver, mostly around the central vein. The mechanism by which the liver lesions are produced is not clear at present. Further work will be necessary in order to prove the *modus operandi*. It is probable that hemorrhage occurs first as a result of fall of blood pressure and stagnation with subsequent vascular injury, and necrosis sets in secondarily. The frequent predominance of the former in those mice which died early seems to support this assumption. Furthermore, the occurrence of ascites, hydrothorax, and pulmonary edema, also speaks for vascular changes as the primary cause. No explanation can be given as to why necrosis does not occur constantly in a given portion of the liver lobule. It is also possible that hemorrhage into liver cell cords is due to decrease in prothrombin as with other hepatic poisons which have been aptly discussed by Quick (21). This phase of the question is being investigated in this laboratory.

Attempts to produce cirrhosis of the liver by repeated administration of small doses of the alkaloids, intravenously, subcutaneously, or orally, have failed. In its place, hypertrophy of the parenchymal cells and their nuclei is uniformly observed. It must be assumed that the mouse responds differently from cattle, for the latter are said to develop liver cirrhosis from eating *Senecio* plants. Davidson (19) using retrorsine also was unsuccessful in producing cirrhosis in rats. He reported endothelial proliferation in the hepatic and sublobular veins of the liver. This was not present in the livers of our mice.

#### SUMMARY

1. The median lethal doses of senecionine, integrerrimine, jacobine, longilobine, and spartiodine, have been determined in mice by intravenous injection. Death occurs in 24-96 hours following medication.

2. Necropsies have been performed on all animals, dead or surviving for a week. Necrosis of the liver, chiefly central, associated with sinusoidal congestion and hemorrhage into cell cords is uniformly observed.

3. Repeated administration of small sublethal doses of the 5 alkaloids, intravenously, subcutaneously, or orally, is followed by hypertrophy of the liver cells and their nuclei. In no instance is cirrhosis of the liver observed.

4. All the 5 alkaloids lower arterial blood pressure of etherized cats, and inhibit the peristaltic movements of isolated rabbits' intestines. Integrerrimine, longilobine, and jacobine cause contractions of isolated guinea pigs' uteri.

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preparations cause a fall in body temperature, while therapeutic doses have no such effect. In rats nonlethal doses frequently produce an anorexia with gradual loss of weight and muscular tone. In both species death appeared to be due to respiratory failure since the heart continued to beat for a considerable time after respiration had ceased.

*Cumulative toxicity.* The effect of repeated daily administration of gramicidin and tyrothricin was determined in a series of 10 dogs. For these studies the preparations were suspended in 5 per cent glucose and administered intravenously in doses of 2 and 4 mgm. per kilogram. Body temperature, heart and respiratory rate, and blood picture (red, white, differential count, hemoglobin and hematocrit) were observed at frequent intervals. The blood samples for analysis were taken prior to the injection of the suspensions.

Daily doses of 2 mgm. of gramicidin or tyrothricin per kilogram caused death in most dogs within 2-8 days. Gramicidin appeared somewhat more toxic than tyrothricin since most deaths following gramicidin administration took place after 2-3 injections whereas 5-6 injections of tyrothricin were tolerated. All dogs developed anorexia and lost weight; the latter effect became more pronounced as the experiment progressed. During and shortly following each injection most dogs secreted excessive amounts of saliva and showed a slight rise in body temperature. Shortly before death the body temperature decreased. No significant change in heart or respiratory rate took place until shortly before death, at which time the heart rate became slow and the respiration shallow.

Daily blood examinations showed that all dogs receiving 2-4 mgm. of tyrothricin per kilogram developed marked leucocytosis. Dogs which tolerated more than 10 consecutive doses of the drug became anemic, the erythrocyte count ranging from  $2.06 \times 10^6$  to  $3.95 \times 10^6$  cells per c.mm. One dog with marked leucocytosis and anemia returned to normal after a period of 2 months during which time no drug had been given. This might indicate that the anemia caused by daily injections of tyrothricin is related to the hemolytic properties, which it can display *in vitro* (5).

Gramicidin had no apparent effect upon the blood picture during the short period which the animal survived. However, equivalent doses of gramicidin might have a similar effect as tyrothricin if the animal could tolerate a larger number of consecutive doses.

*Pathology.*<sup>2</sup> "Upon autopsy there were generalized congestion with degenerative changes in all organs, fatty liver, and an occasional enlargement of the spleen. Ascites was found in most cases. Upon microscopic examination the following changes were found: The congestion in the spleen was most pronounced. If the spleen did not have an open circulation this might have readily been interpreted as hemorrhage into the organ. At times the congestion

<sup>2</sup> We are indebted to Dr. William Antopol of the Newark Beth Israel Hospital for the pathological findings.

appeared to be most pronounced in the perifollicular zone, but this was not a constant finding. The cells in the pulp were very few in those areas in which the cytoplasmic reticulum was present—it appeared to be undergoing severe degenerative changes and necrosis with karyorrhexis."

*Effects on isolated organs.* The effect of gramicidin, tyrocidine and tyrothricin was investigated in the isolated rabbit's intestine and the isolated frog heart. The pH of all solutions was between 7.4-7.6. Loeke's solution was used for the experiments on isolated intestine and Ringer's solution for the Straub heart.

In the isolated rabbit's intestine tyrocidine in doses of 6.4 mgm. per 100 cc. of Locke's solution produced a marked contraction whereas similar doses of



FIG. 1. ACTION OF GRAMICIDIN AND TYROCIDINE ON ISOLATED RABBIT INTESTINE  
2. 32 micrograms Tyrocidine

gramicidin had no effect. This action was reversible since upon removal of the tyrocidine the intestine returned to normal within 15 minutes (fig. 1).

When tested in the isolated (Straub) frog heart, tyrocidine proved again to be the most active of the 3 preparations. The addition of 32 micrograms of tyrocidine suspended in frog ringer decreased both the rate and amplitude of the ventricular beat, and the heart finally stopped midway between systole and diastole (fig. 2). A similar effect was produced with a dose of 264 micrograms of gramicidin.

*Blood pressure and respiration.* The action of gramicidin, tyrocidine and tyrothricin upon the blood pressure and respiration was determined in cats and rabbits anesthetized with urethane; the usual methods of manometric recording and cannulation of the carotid artery were employed. Injections of the above

agents suspended in saline were made into the jugular vein and the blood pressure, respiratory rate and respiratory volume were recorded. All injections were made slowly, averaging approximately one cc. per minute. In most animals gramicidin, tyrocidine and tyrothricin produced a marked fall in blood pressure after the second or third consecutive injection of 1 mgm. per kilogram or more (fig. 3), while single injections, even of large amounts, frequently had no such effect. Some cats tolerated large doses before a drop in blood pressure was observed. In small doses tyrocidine depressed the respiration somewhat whereas comparable doses of gramicidin did not. With lethal doses of tyrocidine the respiration became irregular and stopped shortly before, or simultaneously with, the terminal fall of blood pressure and standstill of the heart.

*Local effects on skin and mucous membranes.* The clinical application of gramicidin and tyrothricin to mucous membranes led us to study their possible irritating properties on such tissues. This was done by instilling saline suspensions of both preparations into the conjunctival sacs of albino rabbits or by

FIG. 2. EFFECT OF GRAMICIDIN AND TYROCIDINE (GRAMINIC ACID) ON THE ISOLATED FROG HEART

injecting 0.1 cc. subcutaneously or intradermally into the shaved abdomen of guinea pigs. No evidence of irritation was observed if concentrations of 500 micrograms per cc. of saline were applied to the cornea for 120 minutes. However, when the dry material was dusted into the eye sac there was marked irritation, characterized by edema and inflammation of the conjunctiva followed by a cloudiness of the cornea. These signs were still present 24 hours after installation. Application of an inert powder (barium sulfate) in a similar manner did not produce such an effect. The subcutaneous or intradermal injections produced local nodules which remained unabsorbed over a period of 5-6 weeks.

**DISCUSSION.** The foregoing data indicate that gramicidin and tyrothricin are more toxic than tyrocidine when injected intravenously and intraperitoneally in mice and rats, whereas none of the preparations is toxic upon single or repeated administration by mouth. The impossibility of preparing true aqueous solutions in these studies is a definite disadvantage and makes the interpretation of data difficult. This is particularly true in experiments in which intravenous administration is employed, for then physical factors such as particle size may influence the results.

The physical properties of gramicidin and tyrocidine limit their application to infections in which local therapy can be employed. In this connection the absence of irritating properties even of fairly concentrated suspensions of these agents is a definite advantage over other antiseptics and may extend the scope of their application. Although the toxicity of gramicidin and tyrothricin is considerably greater than that of most agents of the sulfanilamide group, the

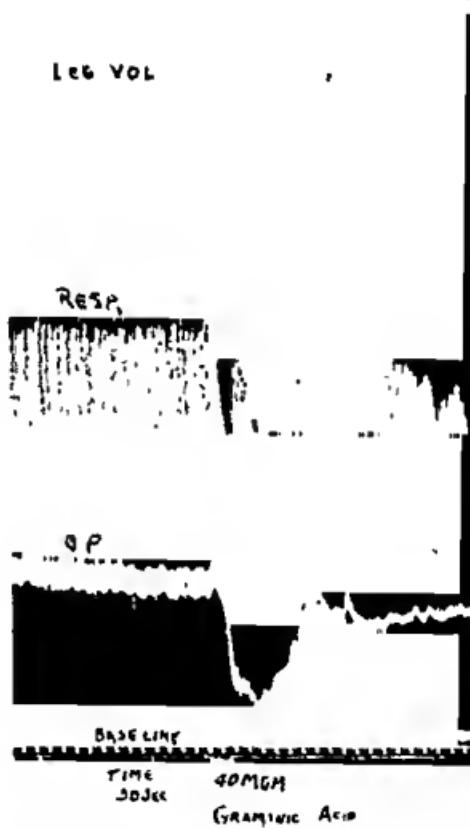


FIG. 3. EFFECT OF TYROCIDINE (GRAMINIC ACID) UPON BLOOD PRESSURE, RESPIRATION AND LEG VOLUME IN A MALE CAT 4.0 KGM. BODYWEIGHT

extraordinary bacteriostatic activity of these compounds would seem to afford a large margin of safety.

Pharmacologically and toxicologically these compounds have no pronounced specific properties. They lower the arterial blood pressure, possibly because of peripheral vasodilatation, and depress the respiration. Death in acute experiments is probably due to respiratory failure, since the heart action continues for some time after the respiration has stopped. Repeated administration of toxic

doses causes degenerative changes in almost all internal organs and marked changes in the blood picture.

In view of the insolubility of these agents it is not unlikely that the effects observed are not caused by a specific pharmacodynamic action, but rather are due to non-specific physical or physico-chemical properties. It is therefore doubtful whether the toxicological results reported in this paper have a direct bearing on the clinical use of these compounds, except that application to deep lacerated wounds might approach the experimental conditions present in intravenous injection. Caution might therefore be in order whenever rapid and direct absorption of these agents into the bloodstream is likely to occur.

#### SUMMARY

1. The acute, peroral, intraperitoneal and intravenous toxicity of tyrothricin, gramicidin and tyrocidine was determined in mice and rats. None of the compounds was toxic when given by mouth. Upon parenteral administration all proved definitely toxic, gramicidin and tyrothricin considerably more so than tyrocidine.

2. Daily parenteral administration of 2 mgm. of gramicidin or tyrothricin per kilogram to dogs caused death within 2-8 days. During this period the dogs lost their appetite and weight and secreted excessive amounts of saliva. Their red blood counts dropped in some cases from 6,000,000 to 3,500,000 per c.mm.

3. In isolated organs as well as *in situ*, tyrocidine produced greater pharmacological changes than tyrothricin or gramicidin, possibly due to its greater solubility in water.

4. None of the drugs has a pronounced specific effect on the respiratory or circulatory system. Large single doses are usually tolerated without any marked effect, while repeated administrations even of small doses cause a fall of blood pressure and impairment of respiration. With lethal doses the respiration stops shortly before the heart.

5. Concentrations of gramicidin suspensions up to 0.5 per cent are not irritating upon instillation into the conjunctival sacs of rabbits. However, application of the dried material produces marked conjunctival irritation and a long-persisting opaqueness of the cornea. When injected subcutaneously or intradermally the preparations remain unabsorbed for a long period of time.

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## STUDIES ON THE PORTAL PRESSURE EFFECTS OF DIGITALIS<sup>1</sup>

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Previous investigators (1, 2, 3) have found that intravenous injection of the tinctures of strophantidin or digitalis, or the proprietary digifolinc (Ciha), brings about an increase in portal venous pressure in dogs anesthetized with morphine and ether. Portal pressure elevation has also been reported (4) following very large doses of digitalis in animals under barbiturate anesthesia. Evidence has been presented which indicates that the observed rise in portal pressure is due to obstruction to venous blood flow through and from the liver, which has been called the hepatic sluice mechanism. It has been proposed (3) that the therapeutic action of digitalis depends primarily upon its effect upon the hepatic sluice mechanism.

There are reasons for doubting that the latter suggestion is correct, an important consideration being that the hepatic enlargement and ascites in cardiac decompensation in man are not increased by digitalis, as they would presumably be if digitalis produced an increase in portal pressure. Rather it is known that the reverse processes occur. Furthermore it is known (5) that in cardiac decompensation in man intravenous injection of suitable doses of pure digitalis glycosides increases cardiac output by from 25 to 40 per cent within thirty minutes after administration, as measured with the Roentgen kymograph technique. Likewise it is known (6) that doses of digitalis hodies, within the therapeutic range, increase myocardial work capacity.

Nevertheless a further study of the conditions under which a portal pressure rise can be obtained with digitalis seemed necessary because such an effect would be important even if it were solely a manifestation of toxic doses. Experiments were therefore designed to ascertain the minimum dose producing the effect. Dogs under nembutal were employed for these experiments and virtually negative results were obtained when pure glycosides were used. No consistent elevation of portal venous pressure was observed with doses in the therapeutic ranges of any crystalline glycoside used, although the tincture of digitalis and the partially purified material digifoline did exhibit an inconstant effect. Because of the difference between these findings and

<sup>1</sup> Aided by Technical Assistance, Project No. 65-1-71-140, Sub-Project 235 W.P.A.

doses causes degenerative changes in almost all changes in the blood picture.

In view of the insolubility of these agents it is observed are not caused by a specific pharmacological effect due to non-specific physical or physico-chemical factors. It is doubtful whether the toxicological results reported bearing on the clinical use of these compounds, lacerated wounds might approach the experimental venous injection. Caution might therefore be exercised in the absorption of these agents into the blood stream.

#### SUMMARY

1. The acute, peroral, intraperitoneal and intravenous effects of gramicidin and tyrocidine was determined in compounds was toxic when given by mouth. It all proved definitely toxic, gramicidin and tyrocidine more than tyrocidine.

2. Daily parenteral administration of 2 mg. per kilogram to dogs caused death within 2-8 days. The dogs lost their appetite and weight and secretions. Their red blood counts dropped in some cases to 1,000,000 per cu. mm.

3. In isolated organs as well as *in situ*, tyrocidine produced more toxicological changes than tyrothricin or gramicidin, and is soluble in water.

4. None of the drugs has a pronounced specific effect on the circulatory system. Large single doses are without marked effect, while repeated administration causes a marked fall in blood pressure and impairment of respiration. The heart action stops shortly before the heart.

5. Concentrations of gramicidin suspensions, tating upon instillation into the conjunctival sac or upon application of the dried material produces marked opacity of the cornea. After instillation intradermally the preparations remain unchanged.

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There are reasons for doubting that the latter suggestion is correct, an important consideration being that the hepatic enlargement and ascites in cardiac decompensation in man are not increased by digitalis, as they would presumably be if digitalis produced an increase in portal pressure. Rather it is known that the reverse processes occur. Furthermore it is known (5) that in cardiac decompensation in man intravenous injection of suitable doses of pure digitalis glycosides increases cardiac output by from 25 to 40 per cent within thirty minutes after administration, as measured with the Roentgen kymograph technique. Likewise it is known (6) that doses of digitalis bodies, within the therapeutic range, increase myocardial work capacity.

Nevertheless a further study of the conditions under which a portal pressure rise can be obtained with digitalis seemed necessary because such an effect would be important even if it were solely a manifestation of toxic doses. Experiments were therefore designed to ascertain the minimum dose producing the effect. Dogs under nembutal were employed for these experiments and virtually negative results were obtained when pure glycosides were used. No consistent elevation of portal venous pressure was observed with doses in the therapeutic ranges of any crystalline glycoside used, although the tincture of digitalis and the partially purified material digisoliae did exhibit an inconstant effect. Because of the difference between these findings and

<sup>1</sup> Aided by Technical Assistance, Project No. 65-1-71-140, Sub-Project 235 W.P.A.

those of others, we have re-investigated the problem, with particular reference to the anesthetic agent and the digitalis body employed.

**METHODS.** Dogs were anesthetized either with nembutal (35-40 mgm. per kgm.) given intraperitoneally, or with morphine (20 mgm. per kgm.) subcutaneously supplemented with ether inhalation. Carotid, portal and right atrial pressures were recorded by optical manometric methods. Glass cannulae were inserted into the splenic vein directed toward the portal vein, into the right atrium through the jugular vein, and into the carotid artery. Connections were made to Gihson glass spoon manometers, as described by Kubicek, Sedgwick, and Visscher (7), by means of rigid tubing, mainly lead, connected to glass with heavy fibre tubing. The arterial manometer system was filled with 4 per cent sodium citrate solution, and the venous manometer systems were filled with isotonic saline solution containing 0.01 per cent heparin (Hynson, Westcott, and Dunning) to prevent clotting. Three-way stopcocks in the venous manometer systems permitted easy flushing under pressure with one to two cc. of the heparin-saline solution into the cannulae tips at intervals. The venous manometers were calibrated against water, and the arterial against mercury. Intravenous injections were made through a cannula in the femoral vein. Materials were injected by syringe into the rubber tubing of a burette system and flushed into the vein with 4 cc. of isotonic NaCl solution.

**RESULTS.** The effects of single and repeated injections of digitalis into 33 dogs have been studied in this series of experiments. The main results are summarized in tables 1 and 2. Unless otherwise noted the figures given refer to the effects of the single initial dose of the substance under study. Changes in portal and right atrial pressures of less than 1.5 cm. H<sub>2</sub>O in either direction are recorded as zero, since fluctuations of that order occur spontaneously and are too small to be of appreciable hemodynamic significance. Changes in carotid pressure of less than 4 mm. Hg are similarly treated.

Upon inspection of table 1 it will be seen that with the exception of one instance with digitoxin (Digitaline Nativelle) none of the crystalline glycosides employed caused an increase in portal venous pressure in doses from 8 to 40 per cent of the lethal, when the dogs were anesthetized with nembutal. The arterial blood pressures were ordinarily initially about 120 mm. Hg. In most instances the arterial pressures were elevated by the first digitalis injection but the further course of the arterial pressure was variable. It was noted that on repeated fractional doses, 8-12 per cent of the lethal, at 10 minute intervals, the portal pressure tended to fall until 50 to 90 per cent of the lethal amount was injected, when the portal pressure ordinarily rose. The pre-lethal rise in portal venous pressure was the most consistent finding in the series of experiments under nembutal, in confirmation of Gold and Cattell (4).

When morphine and ether were employed for anesthesia an elevation in portal pressure was found with ouabain and lanatoside C, in doses which did not produce such an effect under nembutal. However the tension rise was transient. In every case in which less than 25 per cent of the lethal dose was injected the portal venous pressure had returned to (usually below) the pre-injection level with 20 minutes.

TABLE 1  
*Pressure changes on administration of crystalline glycosides*

NUMBER OF EXPERI- MENTS*	GLYCOSIDE	DOSE <sup>†</sup> , PER CENT LETHAL	ANESTHETIC AGENT	PORTAL PRESSURE CHANGE†	ATRIAL PRESSURE CHANGE	CAROTID PRESSURE CHANGE†
						cm. H <sub>2</sub> O
1	Digitoxin	8	Nembutal	+3	0	+6
2	Digitoxin	8	Nembutal	0	0	+15
3	Digitoxin	80‡	Nembutal	-3	0	+7
1	Ouabain	8	Nembutal	0	0	+4
1	Ouabain	80‡	Nembutal	+3	0	+12
2	Ouabain	22	Morphine ether	+4	0	+12
2	Ouabain	44†	Morphine ether	+15	0	+55
1	Lanatoside B	8	Nembutal	0	0	+4
1	Lanatoside B	70‡	Nembutal	0		-30
7	Lanatoside C	8	Nembutal	0		+11
1	Lanatoside C	23	Nembutal	0	+2	+11
9	Lanatoside C	40‡	Nembutal	0		-4
7	Lanatoside C	80‡	Nembutal	+4		-25
1	Lanatoside C	22§	Morphine ether	-3	0	+5
1	Lanatoside C	22¶	Morphine ether	+10		+55

\* Except where otherwise indicated the doses shown were the first injections into individual animals.

† Mean maximal change where the number of experiments was 2 or more.

‡ Fractional doses in dogs given only 1 type of glycoside.

§ Following 22 per cent of the lethal dose of Ouabain.

¶ Following 44 per cent of the lethal dose of Ouabain.

|| Not recorded.

TABLE 2

*Mean pressure changes on administration of tincture of digitalis and digifoline*

NUMBER OF EXPERI- MENTS	DRUG	DOSE, PER CENT LETHAL	ANESTHETIC AGENT	PORTAL PRESSURE CHANGES		CAROTID PRESSURE CHANGES
				cm. H <sub>2</sub> O	cm. H <sub>2</sub> O	
1	Tincture	22	Nembutal	0	0	-10
1	Tincture	45	Nembutal	+10	0	+18
2	Tincture	20	Morphine-ether	+8	0	+20
6	Digifoline	8	Nembutal	+3*	0	+4
7	Digifoline	19	Nembutal	+2†	0	+15

\* 1 dog showed change less than 1 cm. H<sub>2</sub>O.

† 4 dogs showed changes less than 1 cm. H<sub>2</sub>O.

Table 2 presents the observed data concerning tincture of digitalis and digifoline. In marked contrast to the crystalline glycosides, these materials produce portal pressure elevation in a majority of instances, even in dogs

anesthetized with nembutal. In confirmation of previous investigators we find the tincture of digitalis produces a marked, though transient portal pressure rise when 30 per cent of the lethal dose is administered under ether.

During the course of these studies it was noted that Hynson, Westcott and Dunning heparin regularly produced a transient rise in portal pressure when injected in amount (20 mgm. per kgm., 5 units per mgm.) adequate to render blood incoagulable. Connaught heparin did not exhibit this property when comparable or larger anticoagulant doses (0.3 cc per kgm., 1000 units per cc.) were injected. Because the more active material (Connaught) had no portal pressure effect the basis of the reaction to the other commercial preparation was not investigated further. Heparinization with material from either source was not found to influence the portal pressure reaction to digitalis bodies.

**DISCUSSION.** The portal pressure increase which others have observed following digitalis body injection and ascribed to constriction of hepatic vessels, could exert a profound hemodynamic effect upon the heart and circulation under appropriate circumstances. However, in order to permit the conclusion that digitalis bodies generally act therapeutically through constriction of the hepatic sluice mechanism it would be necessary to have proof: (1) That all cardio-active glycosides have such an effect in therapeutic doses, and (2) that the effect persists long enough to "unload" the heart effectively.

The evidence presented in this paper is against both of these points. First, we have shown that none of four crystalline glycosides studied possesses the property of consistently elevating portal venous pressure in barbitalized dogs, under circumstances in which the tincture of digitalis and digifoline do produce such an effect. The barbiturates are not known to interfere with the therapeutic action of digitalis glycosides. Their therapeutic effects have been studied under various barbiturates (6). Further, it is known (8) that the toxicity of digitalis bodies both for cats and dogs is greater under ether than under nembutal, the difference being approximately 50 per cent. It is not impossible that the increased sensitivity to digitalis under ether is due to a greater peripheral vascular effect of digitalis bodies in toxic doses in etherized animals. It should be noted that the lethal dose of at least one digitalis glycoside, lanatoside C, for the dog is identical under nembutal anesthesia and in the unanesthetized state (8). There is, therefore, some evidence that the barbitalized animal approximates the normal unanesthetized state more closely with respect to digitalis action than does the etherized animal.

As for the second point, even if for the moment one ignores the fact that under the influence of barbiturates the effect is not seen at all with crystalline glycosides, one can consider the cases in which there is evidence of hepatic vascular constriction. Digifoline produced a rise in portal pressure with doses between 8 and 19 per cent of lethal, that is, in doses of therapeutic importance, in 8 of 13 trials. However, neither with digifoline or the tincture

under nembutal, nor with any drug under ether, was the duration of the portal pressure rise greater than 20 minutes when the dose injected was less than 25 per cent of the lethal. The average duration of the rise in portal pressure was less than 10 minutes. Furthermore the long-time trend of portal pressure is a slight decrease rather than an increase until 50 to 90 per cent of the lethal amount has been injected. It is difficult to comprehend a mechanism which, by taking a small load off the heart for 20 minutes, would set in motion processes which would rapidly reverse the phenomenon of heart failure over a period of days thereafter. It is our opinion that the short duration of the rise in portal pressure renders extremely unlikely the possibility that it reflects an action (hepatic sluice closure) of primary importance to the therapeutic action of digitalis, which is characterized by the great duration of its action. It is more likely that the portal pressure effect, when it occurs, is a side reaction of the drug, of no importance to the main therapeutic effect upon the heart (6).

#### CONCLUSIONS

1. The effects of several digitalis bodies upon portal venous pressure have been studied in the dog in relation to type of anesthesia and dose of drug.
2. Intravenous injection of the crystalline glycosides, digitoxin, ouabain, lanatoside B and lanatoside C produced an elevation in portal venous pressure in doses from 8 to 44 per cent of lethal in only 1 instance in 23 trials, in harhitalized dogs. Under morphine and ether such an effect occurred in 5 out of 6 trials.
3. Tincture of *Digitalis purpurea* and digifoline (Ciha) produced portal pressure rises in 9 of 15 harhitalized dogs. The previously reported portal pressure rise in morphine-ether anesthetized dogs produced by tincture of digitalis has been confirmed.
4. The duration of the portal venous pressure rise produced by doses of digitalis bodies, 25 per cent or less of the lethal dose, was never greater than 20 minutes, under the conditions of these experiments.
5. It is pointed out that the facts presented concerning the elevation in portal venous pressure by cardiac glycosides are not consistent with the view that portal stasis is an essential element in the mechanism of digitalis action.

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When morphine is added to normal minced muscle *in vitro* the oxygen consumption is increased. Observations were made to determine whether a similar effect could be obtained with muscle from chronically morphinized rats. The results are presented in table 1. Added morphine produces an absolute increase in oxygen uptake which is quantitatively similar to that

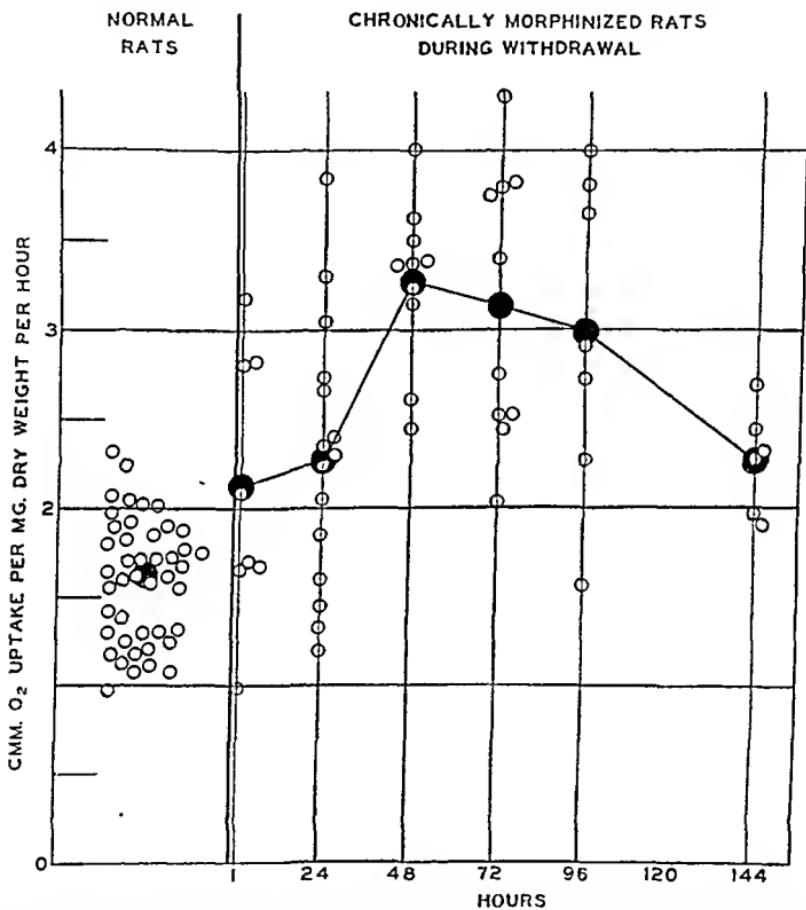


FIG. 1. OXYGEN CONSUMPTION OF MINCED SKELETAL MUSCLE OF NORMAL RATS AND CHRONICALLY MORPHINIZED RATS AT VARIOUS INTERVALS DURING WITHDRAWAL  
Each unfilled circle represents  $Q_{O_2}$  on muscle from one animal

which occurs in normal muscle even though the percentage increase is smaller due to the greater  $Q_{O_2}$  of the chronically morphinized muscle.

In order to establish preliminary data which might serve as a point of departure for the subsequent identification of the oxidative systems involved during chronic morphine poisoning, the action of two commonly used inhibitors, malonate and azide, has been determined.

The addition of 0.02 M malonate produces about the same percentage inhibition of oxygen uptake of both chronically morphinized and normal muscle (table 2). As a result the absolute reduction of oxygen consumption is greater in chronically morphinized muscle since the initial rate of oxygen consumption is higher.

TABLE 1

*Increased oxygen utilization of minced skeletal muscle from normal and chronically morphinized rats resulting from the addition of 0.12 per cent morphine*

	TIME AFTER LAST INJECTION <sup>a</sup>	NUMBER OF ANIMALS	MEAN $Q_{O_2}$	MEAN MORPHINE EFFECT
Normal		42	1.57	+0.58
Chronically morphinized	1 hour	4	2.26	+0.43
	24 hours	15	2.27	+0.46
	48 hours	6	3.03	+0.36
	72 hours	8	3.20	+0.68
	96 hours	2	2.13	+0.00
	6 days	4	2.31	+0.42
	12 days	1	1.98	+0.00

TABLE 2

*Effect of malonate and azide on the oxygen consumption of minced skeletal muscle from normal and chronically morphinized rats*

INHIBITOR	CONDITION OF ANIMALS	NUMBER OF ANIMALS	TIME AFTER LAST INJECTION <sup>a</sup>	MEAN $Q_{O_2}$	MEAN INHIBITION	MEAN PERCENTAGE INHIBITION
Sodium malonate (0.02 M)	Normal	5		1.58	-0.79	-50
	Chronically morphinized	2	1 hour	2.25	-1.24	-55
		5	24 hours	2.10	-1.31	-60
		2	48 hours	3.38	-2.24	-66
		2	72 hours	3.76	-2.25	-60
		2	96 hours	2.14	-1.28	-60
		1	12 days	1.98	-1.28	-65
Sodium azide (0.01 M)	Normal	4		1.26	+0.07	+5
	Chronically morphinized	6	1 hour	2.07	-0.70	-38
		6	6 days	2.26	-0.70	-31

The oxygen uptake of a normal muscle mince is not altered significantly by the addition of 0.01 M sodium azide (table 2). On the other hand, this compound produces a 30 to 40 per cent reduction in the oxygen uptake of chronically morphinized muscle so that the final values for  $Q_{O_2}$  now lie within the range found for normal muscle.

A few experiments were conducted with D-140 (1-methyl-4-phenyl-piperidine carbonic acid ethyl ester),<sup>2</sup> a compound which is stated to have a spasmolytic action on smooth and skeletal muscle. In the concentration used (0.12 per cent), it reduces the oxygen uptake of both normal and chronically morphinized muscle. These results are somewhat similar to those obtained with malonate.

Barbour, Hunter and Richey (4) reported an increased hydration of the blood during morphine withdrawal and state that a similar condition probably obtains in tissues in general. Even though such were the case it would not affect the significance of the results presented here since the  $Q_{O_2}$  is expressed on a basis of dry weight. The data presented in table 3 indicate that no significant change in water content of skeletal muscle occurred in these animals during withdrawal.

TABLE 3  
*Water content of skeletal muscle of normal and chronically morphinized rats*

	HOURS OF WITHDRAWAL	NUMBER OF ANIMALS	WATER CONTENT	
			Mean	Range
			per cent	per cent
Normal rats		42	75.8	71.0-77.7
Chronically mor- phinized rats	1	8	76.5	74.7-78.3
	24	15	75.2	70.8-77.7
	48	10	75.2	73.6-76.9
	72	10	75.9	74.1-76.8
	96	7	75.4	74.4-76.9
	144	6	74.6	74.0-75.3
	12 days	1	76.2	

DISCUSSION. The results presented here represent clear-cut evidence that a fundamental change in functional activity occurs in certain tissues during chronic morphine poisoning which is demonstrable even after these tissues have been isolated from their humoral and nervous connections. Whereas the full significance of this observation and its relation to addiction is not entirely apparent at the present time, it would seem to be more than a coincidence that a curve which represents the increase in oxidative metabolism of skeletal muscle of the chronically morphinized rat during withdrawal should parallel almost exactly in its time relationships one representing the intensity of the abstinence syndrome in the dog (5, 6), monkey (7, 8), and man (3).

These results support in a general way a working hypothesis which we outlined in an earlier paper (1). It is visualized that morphine exerts a sustained and reasonably uniform accelerating action on oxidations in skeletal

<sup>2</sup> Alba Pharmaceutical Company. Furnished through the courtesy of O. W. Barlow.

muscle throughout the course of addiction, but that during maintenance this effect is not detected by changes in bodily functions since the depressant action of the drug on the brain masks this peripheral effect. The specific evidence which lends the greatest support for this concept is the result obtained on chronically morphinized muscle taken one hour after the last dose of morphine. Whereas it must be admitted that no final conclusion is warranted until these results are confirmed, since the number of animals is quite small, the mean  $Q_{O_2}$  of muscle at the one-hour-period is considerably higher than that obtained on normal muscle.

It is quite obvious that the curve shown here, which represents the rate of oxidations in skeletal muscle during withdrawal, would not parallel a curve which could be drawn representing the detoxication or elimination of morphine from the tissues of the same animal over an identical time period. As a matter of fact, a major portion of the drug present in the body at the time of withdrawal would have been eliminated by the seventy-second hour, at which time the rate of oxygen uptake in chronically morphinized muscle is still near the peak of the curve. If the increase in oxygen uptake which occurs when morphine is added to normal muscle in acute experiments bears any connection to the increase in oxygen uptake above the normal level which occurs uniformly in chronically morphinized muscle during withdrawal, the exact relationship is not clarified by the foregoing facts. Nor is it easy to understand why the addition of morphine in acute experiments produces the same absolute increase in oxygen utilization in chronically morphinized as in normal muscle even though the initial  $Q_{O_2}$  of morphinized muscle is nearly twice as great. This latter observation might suggest that in both instances the excess oxygen utilization was due to the oxidation of morphine itself, a concept which we can neither prove nor entirely disprove at this time. The meager evidence which is available at this time indicates that such is not the case. It is hard to conceive of the increased  $Q_{O_2}$  of chronically morphinized muscle as being due to the oxidation of morphine already present in tissues since it increases and remains high as the morphine content of the tissues diminishes.

Stannard (9) has presented evidence which indicates that azide has a definitive inhibitory action on what he terms activity metabolism, although it does not affect resting metabolism. If this view is correct, then the increased oxygen uptake of chronically morphinized muscle must be related to activity since a concentration of azide which is without effect on normal muscle reduces the oxygen utilization of chronically morphinized muscle until the final  $Q_{O_2}$  falls into the range obtained for normal muscle.

Whereas an azide-sensitive system which is not active in normal resting muscle appears in chronically morphinized muscle, the data available indicate that the activity of the malonate-sensitive fraction of respiration is increased in proportion to the increase in total respiration. Thus malonate produces

a greater absolute inhibition in chronically morphinized than in normal muscle, yet the percentile inhibition is the same. It appears, therefore, as if the malonate-sensitive fraction of respiration is affected quantitatively but not qualitatively by chronic morphine poisoning.

#### SUMMARY

The rate of oxygen consumption of minced skeletal muscle from normal rats has been determined and compared with similar data from chronically morphinized rats sacrificed at 24 hour intervals during the first week of withdrawal.

The mean  $Q_{O_2}$  of chronically morphinized muscle from 56 animals calculated without regard to time of withdrawal was 61 per cent greater than the corresponding value for 44 normal rats. The rate of oxygen consumption was greater than normal even one hour after the last dose of morphine and increased rapidly during the first forty-eight hours until at this period it was double the normal level. This high rate persisted until the 96th hour, then gradually subsided but remained above the normal mean at the sixth day. A curve representing these levels of oxygen utilization during the first week of withdrawal parallels almost exactly in its time relationships one representing the intensity of the abstinence syndrome.

The addition of morphine produced an increase in oxygen uptake which was the same in chronically morphinized as in normal muscle regardless of the existing level of metabolism.

Azide, in a concentration which has no significant effect on normal muscle, abolished the increment in oxygen uptake which results from chronic morphine poisoning.

Since malonate produced the same percentage inhibition in chronically morphinized as in normal muscle, irrespective of the level of oxygen consumption, it appears as if the malonate-sensitive fraction of respiration is affected quantitatively rather than qualitatively by chronic morphine poisoning.

The authors are indebted to James K. Theisen for assistance in this study.

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# EXPERIMENTAL COMPARISON OF SEVERAL ALKYL MERCURIC CHLORIDES AS "SKIN STERILIZING" AGENTS<sup>1</sup>

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Among the antiseptics in common use, several organic aryl derivatives of mercury are prominent. The alkyl derivatives of mercury, on the other hand, have been almost completely neglected in the many published studies of antisepsis. Many of these substances are well known to chemists. That they would have bactericidal properties seemed highly probable.

A careful search of the literature revealed that apparently the only published study of the *in vivo* bactericidal action of this type of mercurial was that of Bass (1) who found that the skin-sterilizing power of *n*-propylmercuric chloride was greater than that of several other common antiseptics. The present paper describes the results of a similar careful comparison of several simple alkylmercuric chlorides. As the *n*-propyl derivative is included, these results can be compared directly with those obtained by Bass.

**EXPERIMENTAL.** The compounds studied were prepared in this laboratory by well-known methods: the methyl- and ethylmercuric halides from the corresponding dialkylmercury (2), the higher derivatives through the corresponding Grignard reagent (3). The products were obtained pure by two or three recrystallizations from ethanol. The test solutions were prepared by dissolving the crystalline mercurial in 95 per cent ethanol and diluting to the desired concentration. It is somewhat easier first to dissolve the substances in a small volume of acetone and then to dilute with alcohol and water.

The comparisons of the skin-sterilizing powers of these substances were made by the *in vivo* "skin-graft" technique using rabbit skin as described by Bass (1). Special care was taken to test for haemostatic action. The results are summarized in table 1.

**DISCUSSION.** The data show that all solutions of 0.0036 molar concentration in 70 per cent ethanol produced a high percentage of sterilization. The differences between these figures are not significant. The data for the more dilute solutions show that the use of 25 per cent ethanol almost eliminated the sterilization factor due to the solvent. The fact that the *per cent sterile* is about the same

<sup>1</sup> The funds for carrying out this work were kindly given to us by the Mallinckrodt Chemical Works.

for the 0.0036 molar (1:1000) propylmercuric chloride in the two solvents indicates that there is a considerable overlapping of the action of the solute and the 70 per cent ethanol. That the higher concentration of the solute should be more effective was to be expected; experimentally, it has been shown by Smith, Czarnetzky and Mudd (4) that the effectiveness of various mercurials against microorganisms is directly proportional to the amounts of mercury present relative to the numbers of the bacteria and/or the amounts of protein. Since in our experiments the several alkylmercuric chlorides were made up in equimolar solu-

TABLE 1

ALKYL-MERCURIC CHLORIDE	M.P., CORRECTED	SOLVENT (ETHANOL-WATER)	CONCENTRATION (MOLAR)	NUMBER OF RABBITS	MEDICATED SKIN SNIPS		STANDARD OF COMPARISON*		DIFFERENCE†
					Total number	Per cent sterile	Total number	Per cent sterile	
Methyl.....	174-5	25	0.0018	5	60	31.6	60	1.6	30.0
Ethyl.....	195-6	25	0.0018	5	60	33.0	60	1.6	31.4
<i>n</i> -Propyl.....	144-5	25	0.0018	4	48	41.7	48	0.0	41.7
<i>n</i> -Propyl.....		25	0.0036	7	84	84.5	84	1.2	83.3
Methyl.....		70	0.0036	6	72	91.7	72	50.0	41.7
Ethyl.....		70	0.0036	6	72	83.3	72	47.2	36.1
<i>n</i> -Propyl.....		70	0.0036	4	48	81.3	48	27.1	54.2
<i>n</i> -Butyl.....	128-9	70	0.0036	4	48	93.7	48	47.9	45.8
<i>n</i> -Amyl.....	127-8	70	0.0036	7	84	97.6	84	46.4	51.2
<i>n</i> -Heptyl.....	120-1	70	0.0036	4	47	87.2	47	34.2	53.0

\* In each case the standard of comparison was the same as the solvent for the antiseptic.

† The figures in the column headed "Difference" represent the per cent sterile of the medicated skin snips, minus the per cent sterile of the skin snips treated with the control solutions.

From each rabbit three pieces of untreated skin were cultured and invariably gave good growths.

All "cultures" which remained clear after 48 hours incubation were inoculated with from 50 to 1000 organisms obtained from an untreated skin culture. All these flasks developed good growths. Thus bacteriostasis was negligible.

tions, any differences in effectiveness would have been attributable to differences in penetrating power.

Preliminary tests with several other alkylmercury compounds were carried out. A number of the simple *R*-Hg-Br derivatives showed definite bactericidal action, but as their solubilities were lower than those of the chlorides—and they seemed to offer no advantages—they were not studied further. A solution of 0.034 per cent diethylmercury in 70 per cent ethanol was ineffective.

In consideration of the possible clinical use of these compounds for pre-operative skin sterilization, preliminary tests were performed with the 1:1000 solution of propylmercuric chloride. In our experience the normal unbroken human skin

has shown no ill effects from the several times repeated daily application of the solution. No effort to measure directly the antiseptic's effectiveness on human skin has been made.

Favorable results have been obtained in the treatment of several cases of trichophytosis. Occasionally a blister has occurred, probably due to sensitivity of the skin in the infected area, or to the building of a high concentration of the mercurial by repeating the applications as the solvent evaporated.

#### SUMMARY

The compounds  $R\text{-Hg-Cl}$ , in which  $R$  is methyl, ethyl, *n*-propyl, *n*-butyl, *n*-amyl and *n*-heptyl, have been tested as skin disinfectants, using the *in vivo* "skin-graft" technique on rabbit skin. The 0.0036 molar solutions in 70 per cent ethanol all gave a high percentage of sterile skin snips. If the 0.0036 molar (1:1000) solution of *n*-propylmercuric chloride is taken as a standard, and the above results thus compared with those of Bass (1), it is found that solutions of the simple alkylmercuric chlorides are as effective as any and more effective than many of the commonly used antiseptic solutions when compared by this method.

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## IN VITRO ACTION OF SULFONAMIDES ON LYMPHOGRANULOMA VENEREUM VIRUS

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Treatment with sulfonamide derivatives of four virus diseases has been reported; these are trachoma (1), inclusion blennorrhea (2), a (distemper-like) ferret virus disease (3), and lymphogranuloma venereum or lymphopathia. The first observations on the experimental treatment of lymphogranuloma venereum virus infection were those with prontosil (4) in infections of mice and guinea pigs. Sulfanilamide was found to retard the development of the virus in mice (5). This report was later substantiated by the successful treatment of mice with sulfapyridine (6). McKee, Rake, Greep and van Dyke (7) concluded that sulfapyridine and sulfathiazole had an appreciable effect upon the virus of lymphogranuloma venereum in mice.

That accessory growth factors are rendered inutilizable is one of the theories suggested for the mode of action of the sulfonamide compounds (8, 9). Substances antagonistic to the action of sulfanilamide were found in bacterial extracts (10, 11, 12). At about the same time Woods (13) proved that para aminobenzoic acid had a similar inhibiting effect which was believed to be due to interference with the metabolism of the organisms (14). Additional inhibitors appearing or existing in the tissues and fluids of the body were demonstrated by MacLeod (15). From clinical observations on lymphogranuloma venereum and on blennorrhea, Stein (16) and Thygeson (2) were of the opinion that the drug acted directly upon the virus. In view of these facts it seemed advisable to study experimentally the action of the sulfonamides on lymphogranuloma virus.

By *in vitro* experiments we have attempted to determine the virucidal or inhibitory effect of chemotherapeutic substances in direct contact with the virus of lymphogranuloma venereum and to compare the efficacy of certain sulfonamide compounds. Findlay and MacCallum, 1938 (17), attempted similar experiments using decreasing dilutions of the virus with constant amounts of sulfanilamide, without success.

**MATERIAL AND METHODS.** *Virus:* A strain of the virus of lymphogranuloma venereum, B367, was obtained through the kindness of Dr. S. A. Wykoff, from the Lederle Laboratories, Inc. This was inoculated in successive passages intracerebrally until the strain was consistently pathogenic. The animals showed marked loss of weight, ruffled hair, humped back, conjunctivitis and paralysis; death resulted in five to ten days depending on the

amount of virus injected. In order to determine the most appropriate dilution for our experiments, 0.03 cc. of dilutions 1:20, 1:200 and 1:500 were inoculated intracerebrally in mice. After these virus dilutions had been in the refrigerator over night, other groups of mice were inoculated. Mice receiving 1:20 dilutions of the infected mouse brain showed symptoms within three to four days, death occurring within five days; there was no apparent change in virulence of the virus upon standing for 24 hours at ice box temperature. The mice inoculated with the dilution 1:200 showed acute symptoms within 3 to 5 days, death occurring within 7 days. There was a slight decrease in virulence after standing for 24 hours at ice box temperature. With the dilution of the virus 1:500 the incubation period and the severity of the symptoms varied. Our regular passage mice were subsequently inoculated with dilution 1:20. After four or five days, when definite symptoms appeared, they were killed and a dilution of 1:200 brain emulsion was used for the experiment. Routine cultures were made to detect bacterial contamination.

Periodically, Frei antigens (18) were made from brain emulsions of mice showing acute symptoms. These antigens were tested on patients in parallel with antigen made from the brain of normal mice and a Frei antigen obtained from the Lederle Laboratories, Inc. Our virus antigen and the Lederle antigen gave parallel positive tests. The normal mouse brain antigen was negative.

*Mice:* White mice, obtained from the Carworth Farms, Inc., 15-17 grams in weight, were used for the experiments. To guard against a latent virus such as that of Theiler (19) and Traub (20), in our stock mice, the following safeguards were employed. At various intervals 0.03 cc. of a mouse brain emulsion of a normal mouse was inoculated intracerebrally into three mice. Passages were made from these mice on the seventh day and carried for four passages. At no time was there any evidence of virus disease; the passage mice remained normal.

*Sulfonamides:* The drugs employed were sodium sulfanilyl sulfanilate, sulfaguanidine, sulfathiazole and sulfadiazine.

*Method:* To determine changes in the incubation period, the duration of symptoms and the percentage survival, mice were inoculated with virus exposed to the drugs for different periods. Control mice were inoculated with untreated virus. Solutions of 100, 150, and 200 mgm. in 100 cc. of hot distilled water were made of each drug. Sulfadiazine, the least soluble, was tested out only in 100 mgm. to 100 cc. of hot distilled water.<sup>1</sup> After cooling to room temperature, 2 cc. of each of these solutions was placed in sterile test tubes. To each was added 0.1 cc. of 1:10 dilution of an infected mouse brain emulsion, giving a 1:200 dilution of the virus. The mixtures were shaken and placed in the incubator at 37°C. for one hour, then kept at room temperature for one hour. Following this two hour period, 0.03 cc. was inoculated intracerebrally into 6 to 10 mice. The remainder of the mixtures were kept in the refrigerator for twenty-four hours. Thus the inoculation periods were 2, 6 and 24 hours of virus and drug contact.

Control mice were inoculated with the same dilutions of virus in distilled water, incubated and kept under the same conditions and inoculated at the same time. A control group of mice was inoculated with the drug dilutions without virus. The mice were observed for a month.

In all experiments the technic provided for the comparison of the four drugs in the same experiment, on the same day, using the same virus passage for the dilutions so that there was no variation in virulence, incubation period or temperature. At various intervals, dead mice were autopsied. Passages of the brain emulsion into other mice were made to

<sup>1</sup> After cooling to room temperature the concentration, as measured by the Marshall method, was 200 mg. % for sodium sulfanilyl sulfanilate, 190 mg. % for sulfathiazole, 135 mg. % for sulfaguanidine and 30 mg. % for sulfadiazine. At the end of 24 hours at ice box temperature, crystals of the drugs precipitated out and the concentrations were 200 mg. % for the sodium sulfanilyl sulfanilate, 65 mg. % for sulfathiazole, 60 mg. % for sulfaguanidine and 9.7 mg. % for sulfadiazine.

determine whether virus of lymphogranuloma venereum was present and was the only cause of death.

**EXPERIMENTS.** *Sodium sulfanilyl sulfanilate:* Six experiments were performed with this drug. Table 1 shows that the incubation period is somewhat longer in the mice inoculated with the drug-treated virus than in the control mice. A wider range is observed in a comparison of the average duration of illness, which was more than doubled after the two hour contact period and more than tripled after 24 hours of contact. These observations indicate that the virulence of the virus was attenuated by the drug. The 25 per cent survival of the drug-treated mice with no survivals in the controls supports the view that the virus was definitely attenuated but not completely destroyed because all the experimental mice showed acute symptoms of the disease with slight ameliora-

TABLE 1  
2, 6 and 24 hours. Sodium sulfanilyl sulfanilate-exposed virus\*

CONTACT TIME	NUMBER OF MICE		AVERAGE DAYS INCUBATION	AVERAGE DAYS DURATION OF SYMPTOMS	PERCENTAGE OF SURVIVALS
	Experimental	Control			
Hours					
2	49	20	6 4	7 3	25 0
6	41	19	7 5	8 3	29 14
24	45	16	9 7	10 3	49 18

\* The incubation period was from the day of inoculation to the day symptoms of disease appeared. The duration of symptoms is from the day of appearance of symptoms to either death or recovery.

tion in those that survived during the observation period of 30 days. The symptoms did not entirely disappear.

*Sulfaguanidine:* Five experiments were performed with this drug. The effect of sulfaguanidine is very similar to that of sodium sulfanilyl sulfanilate. A similar increase is observed in the incubation period and the average duration of illness of the drug-treated virus mice when compared with the control mice.

*Sulfathiazole:* Five experiments were made with sulfathiazole. There is a definite increase in the average duration of illness and in the percentage survival, 43 per cent (table 3). The recovered mice showed symptoms of the disease with gradual amelioration.

*Sulfadiazine:* Four experiments were performed with sulfadiazine. Attenuation of the virus was observed here also. There was an increased incubation period and the drug-treated virus was lengthened slightly, ultimately disappearing after 24 hours of contact and very

slightly more with longer contact. None of the control mice, inoculated with the drug solutions without virus, showed any untoward effects from the drugs; they remained normal and gained weight.

DISCUSSION. The effect of the drugs upon the virus is shown by the prolongation of the average incubation and duration of symptoms of mice inoculated with virus exposed to the drugs for various periods as compared with the control mice. Tables 1, 2, 3 and 4 show that following the 2-hour contact period, the incubation period for the control mice was 4 days as compared

TABLE 2.  
2, 6 and 24 hours. *Sulfaguanidine-exposed virus*

CONTACT TIME	NUMBER OF MICE		AVERAGE DAYS INCUBATION	AVERAGE DAYS DURATION OF SYMPTOMS	PERCENTAGE OF SURVIVALS
	Experimental	Control			
hours					
2	32	16	5 4	5 3	15 0
6	30	16	6 3	6 3	20 18
24	23	18	8 6	11 3	43 25

TABLE 3  
2, 6 and 24 hours. *Sulfathiazole-exposed virus*

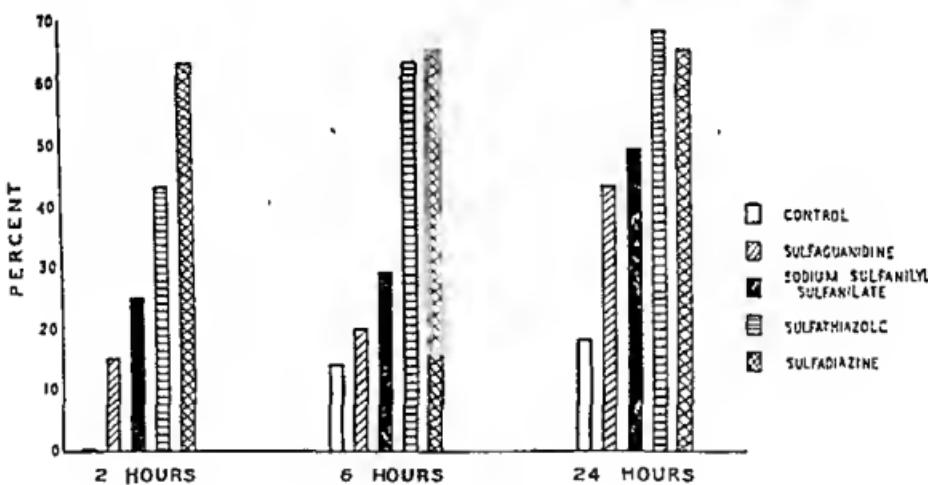
CONTACT TIME	NUMBER OF MICE		AVERAGE DAYS INCUBATION	AVERAGE DAYS DURATION OF SYMPTOMS	PERCENTAGE OF SURVIVALS
	Experimental	Control			
hours					
2	28	16	7 4	10 4	43 0
6	30	16	7 3	14 3	63 18
24	23	12	10 6	12 4	68 25

with 5 to 8 days for the drug treated mice. Similarly, it is found that following a 6-hour period the average incubation for the control mice was 4 days while that of the drug treated mice was 6 to 10 days. Following a 24 hour period, the average incubation for the control mice increased to 7 days, that for the mice inoculated with the drug treated virus was 8 to 12 days. The increase in the incubation period following a 24 hour contact is partly due to the slight loss in virulence of the virus, evidenced by the increase in the incubation time for the control mice. Animals inoculated with virus treated with

sulfaguanidine or sodium sulfanilyl sulfanilate only showed marked prolongation of acute symptoms within 24 hour contact of the virus with these drugs (see tables 1 and 2). The average duration of symptoms in the control mice was 3 days; the corresponding figure for the other mice was 10 to 11 days. Animals inoculated with the virus treated with sulfathiazole and sulfadiazine showed, in most cases, slight symptoms which were markedly prolonged after

TABLE 4  
2, 6 and 24 hours. *Sulfadiazine-exposed virus*

CONTACT TIME	NUMBER OF MICE		AVERAGE DAYS INCUBATION	AVERAGE DAYS DURATION OF SYMPTOMS	PERCENTAGE OF SURVIVALS
	Experimental	Control			
Hours					
2	22	12	8	12	63 0
6	26	12	10	12	65 8
24	18	9	12	13	66 0



GRAPH 1

the 2 hour contact (tables 3 and 4). While the average duration of the control mice was 4 days, that of the other mice was prolonged to 10 to 12 days.

Measured by the percentage of survivals of the mice inoculated with the drug-treated virus (Graph 1) sulfaguanidine is the least effective, resulting in 15 and 20 per cent survivals after the 2-hour and 6-hour contact periods. Similarly, sodium sulfanilyl sulfanilate results in slight effect, 25 and 29 per cent after the first two contact periods. But after the longer period of contact, 24 hours, the

percentage of survivals is increased to 43 and 49 per cent. From these data it appears that sulfaguanidine and sodium sulfanilyl sulfanilate are effective on either a less virulent phase of the virus, as may be surmised from the slight loss of virulence after 24-hour standing, or when in contact for a longer period of time. However, when we deducted from the percentage of survivors after twenty-four hours' contact the percentage of survivors in the control, the same percentage of survival was found, as after 2 hours of contact (tables 1 and 2). Accordingly, the effect is probably produced mostly by the shorter contact, with little change when contact is prolonged.

The same condition was observed in the experiments with the sulfathiazole-treated virus. Deducting the percentage of the control survivals, 25 per cent, from the survivals in the mice inoculated with the drug-treated virus, 68 per cent, after 24 hours of contact, an average survival of 43 per cent resulted (table 3). This is the same as after two hours of contact (Graph I). There were more survivals with sulfathiazole than with either sodium sulfanilyl sulfanilate or with sulfaguanidine. The most effective drug was sulfadiazine,

TABLE 5  
Comparison of amount of drug used and survival after 2 hours

DRUG	AMOUNT OF DRUG IN 2 CC. SOL. mgm.	PER CENT SURVIVAL
Sulfadiazine.....	0.06	63
Sulfathiazole.....	0.38	43
Sodium sulfanilyl sulfanilate.....	0.4	25
Sulfaguanidine.....	0.27	15

The dilution of virus was the same in each instance.

which gave a 63 per cent survival after two hours of contact with little change thereafter (Graph I).

Taking into consideration the amount of the drugs which was contained in the 2 cc. of the saturated solution (note 2) used in the experiments, the smallest amount, 0.06 mgm., was contained in the sulfadiazine solution which gave the highest per cent of survival (table 5). Sulfathiazole solution contained slightly less drug (0.2 mgm.) than the solution of sodium sulfanilyl sulfanilate. The former resulted in a 43 per cent survival while the latter resulted in 25 per cent survival. Sulfaguanidine solution, containing 0.27 mgm., was the least effective drug resulting in a 15 per cent survival.

#### CONCLUSION

Direct *in vitro* contact of sulfonamide compounds with the virus of lymphogranuloma venereum reduces its virulence when subsequently tested in mice. There was no instance of virucidal action but only virus attenuation or inhibition. Sulfadiazine and sulfathiazole are most efficient in this order. Sodium sulfanilyl sulfanilate and sulfaguanidine are less efficient.

Herman Ratish, Chemist of the Littauer Pneumonia Research Fund, assisted with the chemical analyses and Dr. J. J. Levy generously advised during the progress of the work.

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# THE URICOSURIC EFFECTS OF CERTAIN POLYHYDRIC ALCOHOLS AND SACCHARIDES<sup>1</sup>

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In the pursuit of another problem, we had occasion to examine sorbitol in hypertonic solution as a diuretic. Our initial experiments were made with a 50 per cent solution injected intravenously. This solution not only increased the urine volume but also increased markedly the output of uric acid and allantoin. These findings led us to study the effects of other polyhydric alcohols and sugars in a similar fashion; in addition, it seemed desirable to compare the effects of these substances with mercurial diuretics which also increase the output of uric acid (1).

Female dogs with exteriorized ureters were used for these experiments. In this way we were able to collect the urine in hourly periods without manipulation and with the dog standing in a normal position. Although the urine was collected from each kidney and analyzed as such, since the total excretion per hour from the individual kidneys was equal we combined the hourly figures in order to simplify presentation of material. Results are expressed in cubic centimeters or milligrams per kilogram except as otherwise indicated on the charts. One experiment consists of three periods of one hour each. The method of preparing these animals and of collecting the urine has been described in detail elsewhere (2).

The dogs were fed at four o'clock in the afternoon on a standard diet of 150 to 500 grams of meat according to the size of the animal. Two grams of salt were added to the daily diet. Water was allowed *ad libitum*. All experiments were done in the morning. All the drugs were injected intravenously. Water in the water diuresis experiments was given by stomach tube. The strength of the solutions varied with the solubility of the substance used. In the case of dulcitol, we were able to give a super-saturated solution (20 per cent) by maintaining the solution at body temperature until injection. All results are shown in the accompanying charts.

Controls were of two kinds—without any drug, and after the injection of 15 cc. of 0.9 per cent sodium chloride solution. The injections used did not exceed this volume except in the case of raffinose in which a few injections of 30 cc. volume were made. Injection of 30 cc. normal salt solution produced no diuresis in one experiment. It will be seen (fig. 1), that the various constituents remain highly constant under the conditions of this experiment. There is a slight tendency for the three urinary constituents with which we are concerned to decrease in succeeding hours and averages show a distinct decrease. The chart is so plotted that the statistical validity of averages can be seen at a glance. There is a tendency for the urine volume to vary more in the first hour than subsequently and this

<sup>1</sup> We are indebted to the Abbott Company for the Sorbitol and Raffinose used and to the Atlas Powder Company for the Dulcitol and Mannitol.

may be explained by the possibility of the animal having had water just before it was taken out of the cage. However, the differences that are to be discussed are sufficiently large so that this variation does not matter. The excretion was the same from the two kidneys. It will be noted that the uric acid excretion never exceeded 0.09 mgm. per kilogram. Only rarely did the urine volume exceed 15 cc. per kilogram.

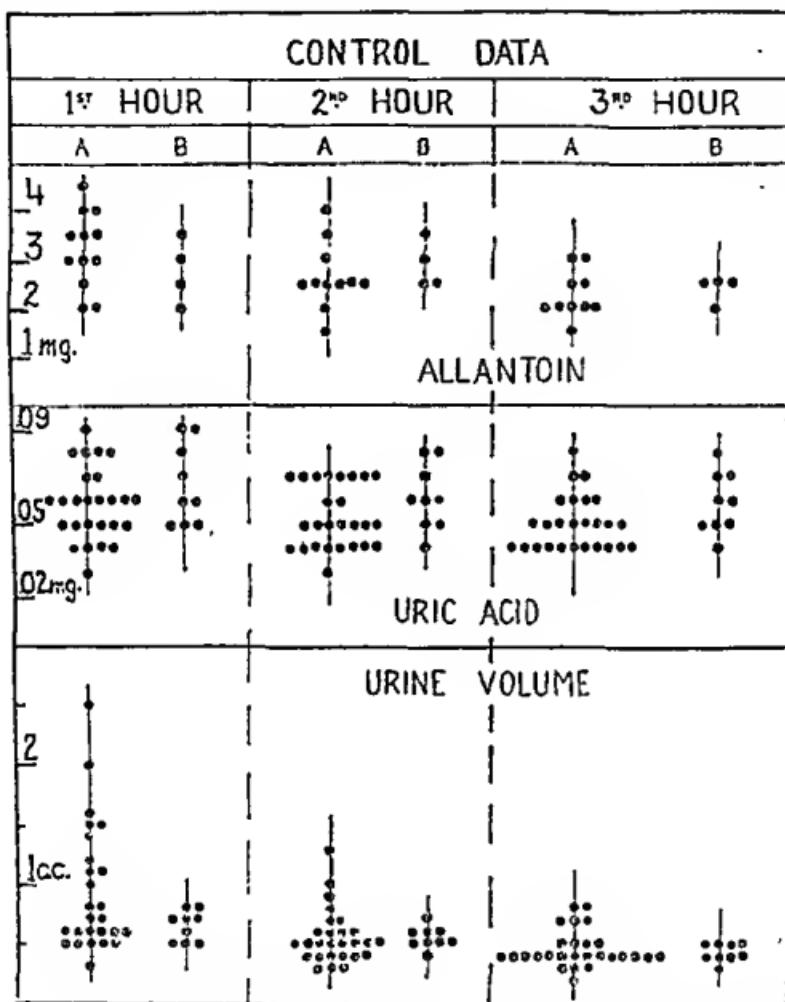


FIG. 1. CONTROL DATA. PLOTTED IN MG.M. OR CC./KGM.

Column A is without any injection and B is after the intravenous injection of 15 cc. normal salt solution.

Sorbitol solution (50 per cent) causes nearly a three-fold increase in uric acid excretion in the first hour (fig. 2). During this same period, 15 cc. of 50 per cent sucrose solution doubles the average uric acid excretion; smaller increases are produced by 50 per cent fructose solution and 20 per cent glycercine. Sorbitol in 20 per cent solution is followed by a less marked but none the less definite

increase in uric acid, whereas 10 per cent sucrose and 10 per cent or smaller concentrations of sorbitol produce less change in uric acid excretion.

After the first hour the action of 50 per cent sorbitol persists significantly though slightly. The action of fructose, glycerine, and smaller quantities of sorbitol and sucrose does not continue beyond the first hour. Fifteen cubic centimeters of 10 per cent raffinose produces a very slight increase in uric acid excretion in the second and third hours. This effect is more pronounced with 30 cc. of 10 per cent raffinose and leads us to suspect that were it possible to give larger doses, a comparable but delayed rise would result. Fifteen cubic centimeters of 50 per cent dextrose, xylose, maltose, galactose, 20 per cent dulcitol

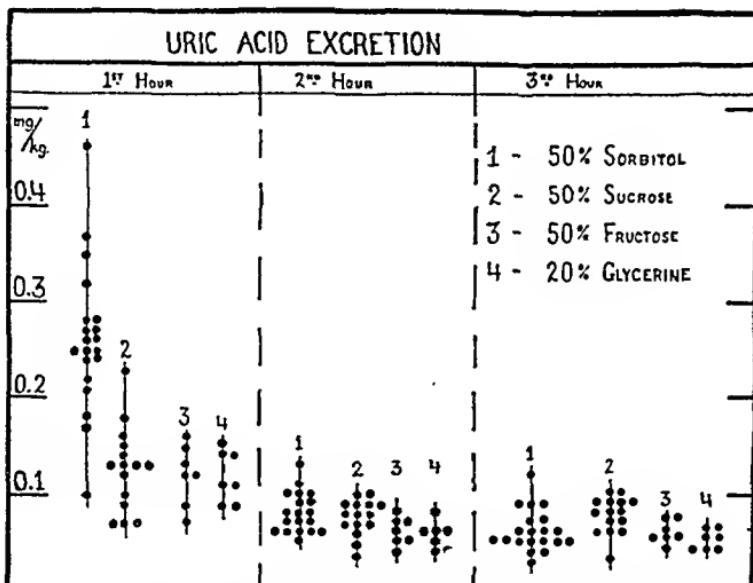


FIG. 2. PROMPT EFFECT OF URICOSURIC SUGARS. SHOWS AMOUNT OF URIC ACID EXCRETED IN THE THREE HOURS AFTER INJECTION OF THE SUGAR

and 40 per cent mannitol have no effect on the uric acid excretion for the three hours following their injection.

Water, when given by mouth in quantities of 100 to 300 cc., causes a diuresis which lasts well into the second hour and in some experiments, into the third hour (fig. 3). The other substances studied produced varying degrees of diuresis though sucrose, sorbitol and xylose were the most efficient in this respect. It is noteworthy that xylose is possibly the most effective diuretic studied though it has no effect on uric acid excretion. In lower concentrations these substances were less diuretic and concentrations as low as 10 per cent were ineffective.

We did not analyze for allantoin those urines containing sugars which on heating with ammoniacal copper solutions hydrolyzed and reduced the copper. Sorbitol, glycerine and mannitol, however, do not reduce the copper solution. We found that the excretion of allantoin was greatly increased by 50 per cent

sorbitol, slightly-increased by 20 per cent glycerine, and unaffected by smaller quantities of sorbitol and mannitol.

The output of urea, total nitrogen and chloride was always proportional to the urinary volume. Some of these sugars reduce the alkaline picrate causing increased color. Therefore, although creatinine was measured on all samples, we can draw no conclusion from the results.

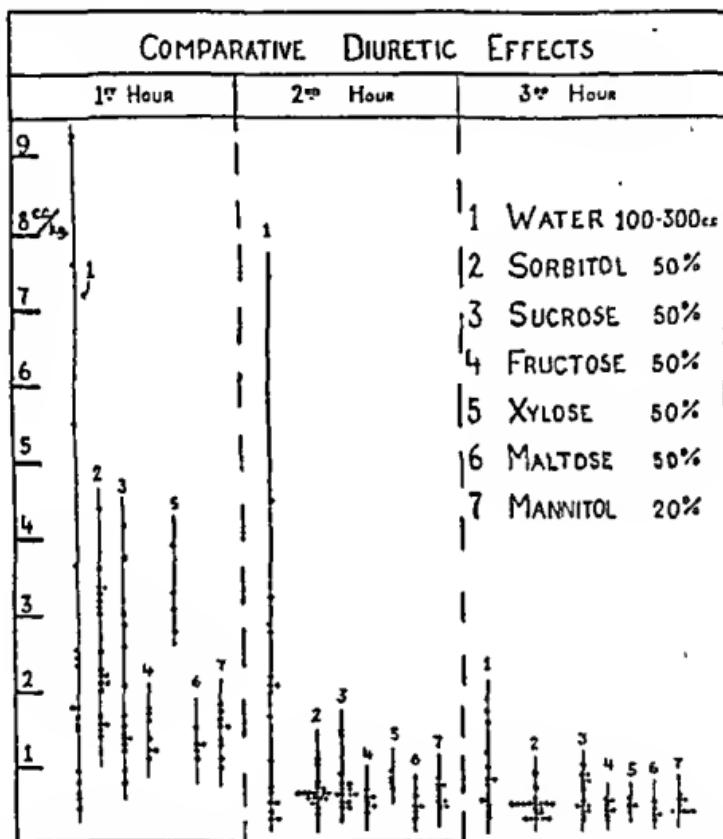


FIG. 3. DIURETIC EFFECTS TO BE COMPARED WITH CONTROLS—FIGURE 1. WATER GIVEN BY STOMACH TUBE, OTHER SUBSTANCES BY INTRAVENOUS INJECTION IN DOSES OF 15 CC. OF THE CONCENTRATION INDICATED

It has been found that 18 hours after eating, the uric acid excretion is solely endogenous (3, 4). Consequently, in all our experiments, we are probably dealing with endogenous uric acid.

The uric acid excretion is augmented by the injection of sorbitol, sucrose, fructose and glycerine. Sorbitol seems to be the most active in this respect in comparison to sucrose and fructose. We thought it advisable not to use glycerine in comparable concentrations because of possible toxicity; therefore, we cannot compare the intensity of its action with the others.

It seemed possible that the increased excretion was a result of the diuresis

causing a washout of uric acid. On comparing the output of uric acid following the injection of these substances with the excretion during water diuresis of equal or greater degree, we found no change in the latter and a large increase in the former.

Further evidence that the increase is not a result of the diuresis is that we have been able to produce an increase in uric acid by a 20 per cent solution of sorbitol

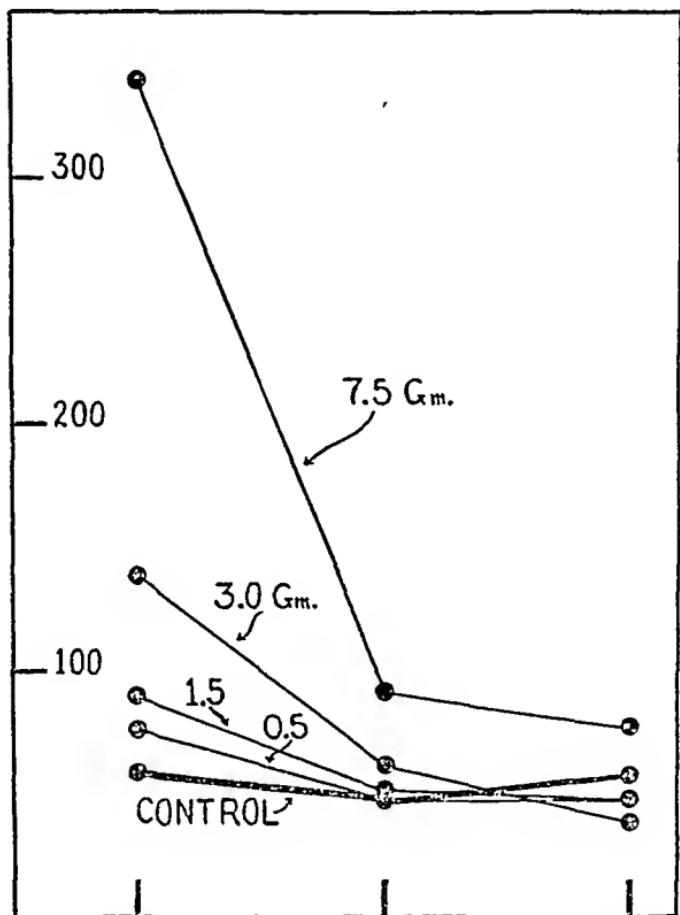


FIG. 4. AVERAGE URIC ACID EXCRETION AFTER DIFFERENT DOSES OF SORBITOL—TIME IN HOURS  
Ordinates plotted in mgm./kgm. per kidney

which produces only a slight change in volume. Smaller concentrations of sorbitol and sucrose which are not diuretic do produce a small increase in uric acid. Since the increase in uric acid appears to be proportional to the dose it is likely that this is a direct effect of the drug (fig. 4).

Simultaneous injections of sorbitol, sucrose, fructose or glycerine with 0.5 mgm. ergotamine or 1 mgm. atropine cause no change in the increased excretion

of uric acid. Denervation of the kidneys produces no change in the action of these sugars. Mares, (3), Smetanka (5), Mendel and Stehle (6), and Ahl (7), all found increased excretion of urine acid after the ingestion of various substances among which were sucrose and glycerine. These observers agree that the increase in uric acid excretion could be explained as the result of the activity of the digestive glands. We find that an increased excretion of uric acid can be obtained by intravenous injection of these two substances. Although we cannot say that the activity of the digestive glands following ingestion of sucrose and glycerine is not a cause of an increase in urine acid excretion, we can say that the activity of the digestive glands is not essential to the increase in uric acid in the case of sorbitol, sucrose, fructose and glycerine. In man, Thomas and Imas (8) have found an increased excretion of urine acid after the ingestion of pentoses; however, Madders and McCance (9) were not able to verify these results.

There seems to be a similarity in the structure of those substances which cause this increased output of uric acid. In all cases, there are two  $\text{CH}_2\text{OH}$  groupings, one on each end of the carbon chain. Substances without this characteristic do not affect the uric acid excretion. Both mannitol and dulcitol, which have two  $\text{CH}_2\text{OH}$  groups so placed and are isomers of sorbitol, do not increase uric acid. From this it is apparent that the optical configuration plays a role in the pharmacologic activity perhaps similar to that seen in various alkaloids in which one optical isomer is active and the other inert.

We can find no direct explanation for the variations in the diuretic responses to these substances. It seems evident that the uricosuric action is not dependent upon the diuresis. Indeed, a few short-interval experiments indicate that the peak of the uric acid excretion comes after the diuresis has begun to subside and that an elevated uric acid excretion is maintained until the urinary volume has returned to normal (fig. 5).

We are unable to explain the increase in allantoin excretion produced by sorbitol and glycerine except to point out that it supports the idea that these substances exert a definite action on the purine metabolism quite apart from their effects as diuretics.

It is evident from the foregoing that an increase in uric acid and allantoin excretion is produced by the intravenous injection of certain sugars and alcohols. Substances having this effect have, in every case, the  $\text{CH}_2\text{OH}$  group at *both* ends of the chain. So far, we have studied trihydric and hexahydric alcohols and certain saccharides containing this configuration. Other related substances do not affect purine excretion. This increase is not related to the "washing out" effect described by Hawk (10). It should be noted that some of these substances produce an increase in urine acid excretion when given by mouth. Mendel and other observers sought to connect this uricosuria with activity of the digestive glands. It is obvious that the prompt effects seen on intravenous injection cannot involve activity of the digestive glands. The recognized relationship between the liver and purine metabolism makes one suspect that this effect is connected with this organ. The mechanism is not at present clear. Renal denervation has no effect. Injections of ergotamine and atropine suf-

cient to block the sympathetic and parasympathetic do not change the effect. Large doses of posterior pituitary extract likewise do not alter the result. Experiments of one dog with an Eck fistula failed to show any change in the reactions to these substances, though of course the initial level of uric acid excretion was higher. It is evident that various substances which might serve to change the uricosuric or diuretic reaction of these carbohydrates are ineffective. We must conclude that the uricosuric action is a function of their chemical structure mod-

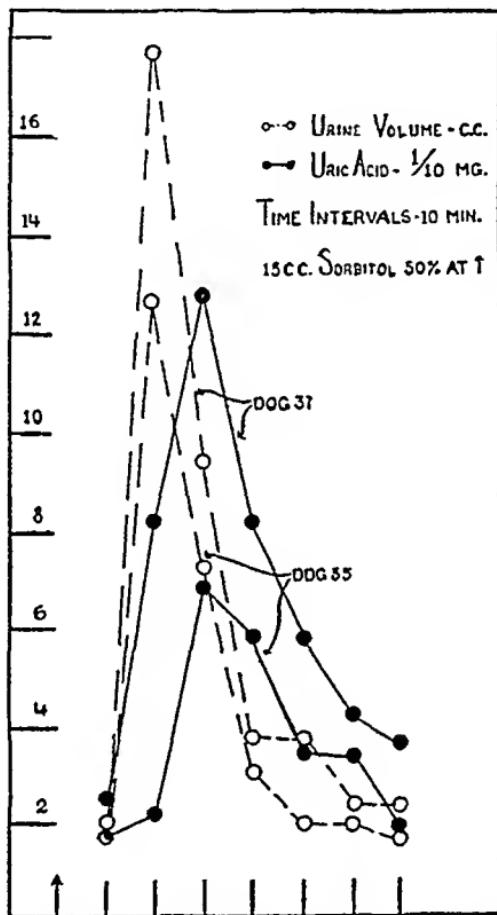


FIG. 5. RELATION BETWEEN THE DIURETIC AND URICOSURIC EFFECT OF SORBITOL

fied by the optical configuration, and is distinct from the diuretic effect which is presumably due to the physico-chemical effects of non-assimilable substances in hypertonic solution.

The observation that salyrgan diuresis is accompanied by an increased uric acid excretion (1), prompted us to use this drug to compare with these carbohydrates in the hope of throwing more light on the mechanism of the uricosuric action. While the diuretic action of salyrgan is comparable to that produced

by the sugars, the uricosuric action of the former in dogs is only trifling as compared with the latter. It was also noted that diuresis produced by salyrgan seemed greater in dogs with one kidney denervated, which suggests that the mechanism of diuretic and uricosuric action of sugars and salyrgan was different, since the action of the sugars was unaffected by denervation, atropine or ergotamine.

#### SUMMARY

1. The effects of intravenous injections of hypertonic solutions of certain polyhydric alcohols and saccharides have been studied in dogs with exteriorized ureters without anesthesia.
2. These drugs produce not only a diuresis but also a marked increase in uric acid and allantoin excretion.
3. All substances causing the increase in purine excretion have the  $\text{CH}_2\text{OH}$  grouping at both ends of the chain.
4. This is not accomplished through a nervous mechanism.
5. It is unlikely that the hypertonicity of the solution per se is the cause of uric acid mobilization, since some hypertonic solutions do not produce this result.

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# A STUDY OF THE TOXIC PROPERTIES OF TUNG NUTS

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The present investigation was undertaken for the purpose of determining what part of the tung nut contains the active toxic principle, in the hope that this substance could be isolated and identified. The toxic nature of tung nuts has been known for a long time, but few instances of experimental study of this property have been reported. Gardner (2) relates an instance of the ingestion of tung nuts by humans. The violent purgative effect which resulted prompted him to study the physiological action of tung oil on rabbits and dogs, in the hope that materials of therapeutic use might be discovered. He found, however, that relatively large doses had only a mild laxative effect. The oil produced no irritation of the intestinal mucosa and no interference with normal functions was observed, although the experiments were continued over a long period of time. His investigation was not extended to other parts of the tung fruit. Carratala (1) has given an excellent description of the symptoms which follow the ingestion of the fruit of the tung tree (*Aleurites fordii*), in describing the poisoning of five workers who had eaten the nuts. None ate more than three of the nuts, but soon showed violent epigastralgia, typical colic pains, bilious vomiting, burning thirst, and profuse diarrhea. As to nervous reactions, there were noted paresthesia, formication, cramps in the legs and arms, nausea, and exhaustion. We have been given an accurate account of the symptoms following the eating of only one tung nut by a student of the University, who mistook it for a Brazil nut. An hour later he felt dizzy and weak and had severe pains in the stomach. Violent vomiting, accompanied by diarrhea, set in immediately, and recurred at short intervals. Attempts to satisfy an unusual thirst seemed to aggravate this condition, which lasted for 6 or 7 hours, after which he was very weak. On the following day he had a headache throughout the morning, but there was no pain. By the third day he had completely recovered.

Godden (3) carried on extensive feeding experiments with tung meal, obtained by solvent extraction, using rats, pigs, and dairy cattle as test animals. The meal which he used was steamed for 40 minutes and dried in air before being used. With these animals a purgative effect was observed, and they lost weight and condition on rations containing meal up to 25 per cent. None of the animals died from the effect of the meal although rats were kept on the ration for 21 days. Godden concluded that the material contained "some substance which makes it unpalatable, and some irritant which has a harmful effect on the mucous membrane of the intestines."

Several instances of dermatitis caused by vapors of heated tung oil have been reported (4, 5), and attributed to the toxic nature of tung nuts.

**EXPERIMENTAL.** The procedure adopted in this work involves a step-wise separation of various constituents of the tung nut with solvents and the testing of the physiological action of the various components so obtained. The object of the separation was the isolation and study of the toxic principle of the nut. The white rat was used as a test animal. The fractions isolated were usually administered in the form of a ration consisting of a mixture of the material and ground dog chow, which was moistened with cane syrup. The resulting dough could be worked into balls of the desired weight, in which form it could be easily fed.

*Physiological action.* *A. Tung nut kernels.* Each of 11 rats was given 15 grams of a ration containing 50 per cent of tung nut kernels. The rats ate this material readily. After three hours a purgative effect was observed in seven cases. After 18 hours this effect was apparent in all of the animals. On the second day 5 of the animals were replaced on their regular diet and they returned to normal in a few days. The other 6 rats were kept on the ration containing tung nut kernels, but after the first day they did not eat the preparation so readily. They became sluggish and apparently very much weakened. On the fourth day one of the animals died. Two died on the seventh day and two more on the eleventh. Only one rat survived to the thirteenth day, when the experiment was discontinued.

*B. Tung oil, separated from (A) by extraction with petroleum ether.* A group of 5 rats was placed on a ration containing 24 per cent of tung oil. The rats ate this preparation readily enough, but remained perfectly normal although they were kept on the ration for 5 days. There was no sign of the purgative effect observed as when the tung nut kernel was fed.

*C. Tung meal, separated from (A) by extraction with petroleum ether.* Each of 5 rats was given 15 grams of a ration containing 19.5 per cent of the oil-free meal. After 14 hours, all of the rats showed definite signs of a purgative effect, and after 38 hours they seemed sluggish and weak. At the end of 62 hours two of the animals were found dead. At this time each of the other three animals was given an additional 5 grams of the food mixture, and at the end of 86 hours none survived.

*D. Tung meal, after extraction of (C) with alcohol.* A group of 5 rats was given daily 10 grams each of a mixture containing 19.5 per cent of tung meal (D). After five days the animals remained perfectly normal and the experiment was discontinued.

*E. Material, obtained from alcohol extract of (C).* About 130 grams of oil-free tung meal (C) was extracted for 10 hours in a continuous extractor with 95 per cent alcohol. The meal remained at 150-160°F. during this time. Upon evaporation of the alcohol a pasty reddish-brown residue was obtained, which became lighter in color and definitely crystalline in appearance when washed with petroleum ether. The yield was 3.85 grams. This solid material was administered orally, in water solution, in doses of 0.3 grams (equivalent to 10.1 grams oil-free

meal) to each of 5 rats. The animals were kept on their regular ration and observed for 24 hours. No physiological effect was observed. The doses were repeated, but again no effect was observed.

The extracted tung meal (*D*) and the material (*E*) were mixed in the same proportions in which they existed in the original oil-free meal (*C*), and tested in the feeding experiments as above. The experiment was continued for 5 days, but none of the symptoms of tung nut poisoning was observed.

*F. Tung meal, after various heat treatments.* A sample of oil-free tung meal (*C*), after heating in a hot air oven at 195°-205°F. for 4 hours, was made up into a ration containing 19.5 per cent of this material, which was fed to a group of 5 rats at the rate of 5 grams each daily. On the second day a slight purgative effect was noted. The experiment was continued for six days, but none of the rats died or showed the sluggishness observed when unheated meal was fed.

The experiment was repeated with meal which had been heated in a hot air oven at 212°-247°F. for 15 hours, with the results that no signs of a purgative effect were observed during the six days that the experiment was continued.

A quantity of oil-free tung meal (*C*) was heated at 230°F. with steam in a pressure cooker for two hours, dried, and tested in the feeding experiments as above. Five rats were given 10 grams each per day for 5 days, but no toxic symptoms appeared.

*G. Press cake.* A ration containing 27 per cent of tung press cake was given to each of 5 rats in 10 gram portions daily. (The press cake is the product obtained from the commercial pressing process. The ground kernels are heated with steam before being pressed.) The rats ate most of the food given them, and after three days, no harmful effects were observed.

**DISCUSSION.** In this investigation it is shown that the decorticated kernels of tung nuts are toxic to rats. When finely ground kernels are included in the food of white rats the animals quickly develop signs of a purgative effect. After some time the animals grow sluggish and weak and finally die.

If the oil is removed from the kernels by extraction with petroleum ether (Skelly Solv "A") before mixing with the food, the toxicity persists. In this case symptoms of poisoning follow more quickly. In an experiment conducted on five rats with oil-free meal, all of the animals died within 86 hours after the first feeding. The extracted oil, after removal of solvent, had no effect upon rats, either when mixed with the food or when administered orally by means of a pipette in doses as large as 1.5 cc.

After the toxicity of the oil-free kernels was established, the material was extracted with alcohol for ten hours, during which time the temperature of the material was 150°-160°F. The residual meal was not toxic, nor was the solid material obtained upon evaporation of the alcoholic extract. The latter material was added to the former in the same ratio that they existed in the meal before extraction, but this mixture also had no physiological effect. The long heating in the presence of alcohol apparently either decomposed the toxic substance or destroyed some enzyme necessary for its formation.

Our experiments show that heating with steam at 230°F. for two hours renders

the oil-free meal non-toxic to rats. Heating in a dry condition apparently requires a longer time to render the material non-toxic, for when the dry meal was heated in a hot air oven at 195°-205°F. for four hours and given to rats, the animals developed slight symptoms of the purgative effect produced by unheated meal but did not grow sluggish or weak. The dry meal was rendered completely non-toxic by heating for fifteen hours at 212°-247°F. in an oven.

In view of the effectiveness of steam upon the highly toxic oil-free meal, the non-toxic nature of the press cake investigated here is especially of interest, as the press cake was obtained from ground kernels which had been steamed before pressing in the commercial plant. The results thus far obtained indicate that a perfectly non-toxic material may be readily obtained as a product of the tung oil industry.

The authors are indebted to the late Dr. Charles E. Coates for helpful suggestions, and to Mr. D. T. Cushing of the Great Southern Lumber Co., Bogalusa, La., who provided samples of tung nuts, press cake, and tung oil.

#### SUMMARY

The toxic principle of tung nuts is not present in tung oil, but is found in tung meal. A method by which this principle may be destroyed is described. An attempt to isolate the toxic material was unsuccessful.

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# THE DELAY IN ONSET OF ACTION OF INTRAVENOUSLY INJECTED ANESTHETICS<sup>1</sup>

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The delay that occurs between the intravenous introduction of certain narcotics and the full development of their action has attracted relatively little attention and has not been satisfactorily explained. The first narcotic found to show this lag was  $\alpha$ -d-glucoclhloralose. Soon after the discovery of the narcotic activity of this drug, the lag was clearly described (1). In the other important class of narcotics having slow onset of action, the barbituric acids, this property was not so promptly recognized. For several years after the introduction of barbital into medicine nearly all of the laboratory and clinical studies were made with the acid form given by mouth. The first reference that I have found to the delay in onset of narcotic action after intravenous injection of a barbituric acid is that of Impens (2), who described it for phenobarbital in 1912. His reference to the lag is casual but unequivocal. Other early references to the slow onset of action of barbituric acids are those of Tiffeneau (3) (barbital, dial, ethyl-allyl-barbituric acid) and of Starkenstein (4) (barbital). Of the other drugs tabulated in this paper as having lags, some have previously been reported to have this property. Others have not. Some have even been described as having immediate onsets (e.g., nostal and nirvanol (5)).

PART 1. *Relation between dose and lag.* For the investigation of the relationship between dose and lag, phenobarbital and  $\alpha$ -d-glucoclhloralose were chosen as representative of the two principal classes of narcotics having lags. Phenobarbital was selected as the representative of the barbituric acids because, of all those with long lags, it gives the quietest anesthesia. The time at which anesthesia is reached is, for that reason, more easily and accurately determined. Moreover, the duration of phenobarbital anesthesia is very long, and the form of the dose-lag relation should be relatively little affected by the inactivation of the drug. Less exactly determined data for barbital indicate that the dose-lag relation is of a similar form to that of phenobarbital. At corresponding doses, the lags are about 1.8 times as great as those of phenobarbital.

<sup>1</sup> This work was supported by a grant from Mallinckrodt Chemical Works. Some of the drugs used were kindly supplied by the following firms: Hoffmann-LaRoche, Eli Lilly & Co., Riedel-de Haen, Sandoz Chemical Works, Sharp and Dohme, and Winthrop Chemical Co.

METHODS. Both drugs were given intravenously to male white mice. Phenobarbital was given as a freshly prepared solution of the sodium salt. The concentrations of the solutions were made proportional to the dose, so that all mice receiving the same drug received the same volume of solution per gram of body weight (phenobarbital, 0.017 cc.; chloralose, 0.03 cc.). The injection of each of the 5 lower doses of phenobarbital occupied  $\frac{1}{2}$  minute (1 minute for the highest dose); each dose of chloralose, 2 minutes.

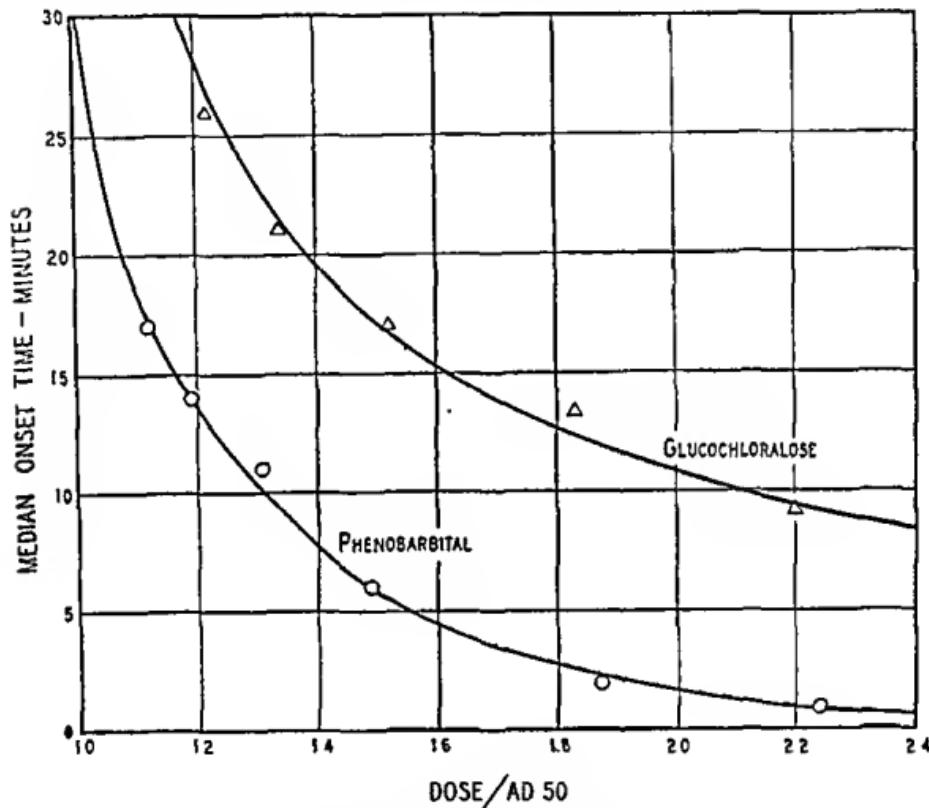


FIG. 1. RELATION BETWEEN DOSE/AD 50 AND TIME OF ONSET OF ANESTHESIA

For each point 25 mice were used. Ordinate—median time between end of injection and onset of anesthesia. Abscissa—ratio of dose to the previously determined median anesthetic dose of the drug (chloralose, 0.032 mgm. per gram; phenobarbital, 0.134 mgm. per gram). The curve fitted to the chloralose data is  $y = 36 \log_{10} \frac{x}{x-1}$ ; that fitted to the phenobarbital data is  $y = 19e^{-2.4(x-1)}(x-1)^{-0.1}$ .

The 150 mice receiving the 6 doses of phenobarbital represented in figure 1 were assigned in groups of 6 at random to the 6 doses. The 6 mice were all dosed on the same day. A similar procedure was used for the 5 doses of chloralose.

The median anesthetic doses, which enter into the expression of dosage used in figure 1, were determined by the same method used for all of the other drugs. (See Part 2.)

The criteria of anesthesia were the same as those described in Part 2.

The results are shown in figure 1. Median rather than mean values of time are plotted because it was thought that this would give a picture of the dose-

time relationship more representative of that which would be found if it could be determined for an individual mouse.<sup>2</sup>

To draw any very definite conclusions from the shapes of the curves would be hazardous.<sup>3</sup> Time-concentration curves for much simpler biological systems have yielded very little information as to the physico-chemical processes involved in the delays. (For a review of this subject, see Clark (7).) The shapes of the two curves are sufficiently different to indicate the need of some caution in assuming that the cause of the lag is the same for the chloraloses as for the barbituric acids. However, either is compatible with the hypothesis that diffusion is an important factor in the delay.

**PART 2. Comparison of the lags of various anesthetics.** As was shown in Part 1, the lag is to a great extent dependent on the dose. For this reason, if the lags of different drugs are to be compared, it is important that doses be used that give comparable depths of anesthesia. In an attempt to fulfill this condition, I have determined the median anesthetic dose of each drug and measured the lag at a dose 1.25 times this value. This ratio gives a dose that proved to be sufficient to anesthetize every mouse with every drug tried.

Drugs were chosen to represent a large variety of chemical classes of narcotics. The majority of those tabulated are derivatives of barbituric acid. The large number of readily available drugs of this group show a wide range of lags. This series affords a good opportunity of studying the relation between structure and lag.

<sup>2</sup> Suppose the dose-lag relation of a population of mice to be represented by the family of curves,  $y = f(x, a)$ , the parameter,  $a$ , determining the position of the asymptote (anesthetic dose). Then the relation between dose and the arithmetic mean of the lags shown by two or more of the curves will not in general be of the form  $y = f(x, a)$ .

The shape of the curves in figure 1 suggests that if the anesthetic doses of a population of mice were normally distributed, the distribution of the lags measured at any dose would not be normal but positively skewed (mean greater than mode). The asymmetry would be more pronounced at lower doses. Calculation of  $g_1$  (Fisher (6), p. 78) shows that the distribution of the times for the lowest dose of phenobarbital departs significantly from normality (positive skewness). For the other 5 doses of phenobarbital, the asymmetry is not significant. On none of the 5 doses of chloralose is there significant asymmetry. Of all the other drugs (Part 2) for which the lags were measured at 1.25 times the median dose, only barbital, nystal, and sandoptol have significantly asymmetrical distributions of lags (all positively skewed).

<sup>3</sup> No particular significance should be attached to the equations used for drawing the curves through the observed points, even though the chloralose equation is of a type that expresses the course of several familiar physical and chemical processes (e.g., the time required for a given amount of solute to diffuse across a thin membrane in accordance with Fick's law). These are the simplest equations giving asymptotes at  $x = 1$  and  $y = 0$  that were found to give a reasonably good fit. (Since the duration of anesthesia is finite with respect to the lag in onset, a curve showing two values of  $y$  for each value of  $x$ , with a tangent at  $x = 1$ , would be a still better representation of reality.) Since a number of different time-concentration relations have been found to fit equilateral hyperbolas and since certain diffusion processes are also represented by such curves, I have tried to fit equilateral hyperbolas to the present data. The data can be fitted rather roughly (considerably less closely than by the curves shown) by these hyperbolas: phenobarbital,  $y(x - 1) = 2.7$ ; chloralose,  $(y - 5)(x - 1) = 5.5$ .

**METHODS.** *Source of drugs.* Those drugs that were not obtainable in a good state of purity either commercially or from the laboratories of the manufacturers were synthesized in this laboratory or isolated from commercial mixtures and purified.

*Administration of drugs.* All drugs were given intravenously to male white mice. The concentrations were usually made such that a mouse would receive about 0.015 cc. of solution per gram body weight, but the low solubility and low activity of some drugs necessitated giving considerably larger volumes of solution. All of the barbituric acids were given as freshly prepared solutions of the sodium salts.

*Criteria of anesthesia.* A mouse was considered anesthetized if it was unable to gain and maintain the standing position after stimulation by repeated pinching of the tail.

*Determination of the median anesthetic dose (AD 50).* For the drugs of tables 1 and 3, two doses were found, one of which would anesthetize between 24 and 50 per cent of the mice, the other between 50 and 76 per cent. Twenty-five mice were used on each of these two doses. The median dose was estimated by interpolation from these two groups on the assumption that the relation between dose and proportion anesthetized is linear over this range.\*

For all of the other drugs (none of which had a lag), only 12 animals were used on each of the two doses. The precision of these doses is consequently less than that of those determined from the larger groups.

The doses of the barbituric acids and hydantoins are expressed in terms of the acid form.

The series of doses shown in the tables were determined over a period of several years and in all seasons. The question of seasonal and other such variations in sensitivity to drugs must be considered in comparing the activities of these drugs.

*Measurement of lag.* For the drugs of tables 1 and 3 a dose 1.25 times the previously determined median anesthetic dose was given. The injection time was usually  $\frac{1}{2}$  minute. Longer injection times were necessary for a few drugs (e.g., 2 minutes for the chloraloses). The time was noted at which the mouse reached the level of anesthesia described above. Times were measured from the end of the injection. Twenty-five mice were used for each drug, the mean time being tabulated (see footnote\*). The lag was always measured within a few days of the determination of the anesthetic dose. The mice used came from the same group from which those used for the dose determination had come. This procedure probably nullified to some extent the effects of the above-mentioned variations on the measured lags.

If in the determination of the median anesthetic dose every mouse that became anesthetized did so within a few seconds of the end of the injection, the use of the higher dose was omitted and the drug classified as having no lag. The onset of action of these drugs may not be quite instantaneous and the drugs may differ slightly among themselves in their rapidity of onset. However, their lags are too short to be determined with any degree of significance by the method described above.

All of the anesthetics that I have found to have any measurable delay in onset of action are to be found in tables 1 and 3. It is to be noted that all of these drugs fall into two chemical classes: the chloraloses, and the 5,5-disubstituted derivatives of barbituric acid and the closely related compound, hydantoin.\* Although I have tested a considerable number of anesthetics belonging to other

\* While this assumption is of course not true, it can lead to no serious error in this range. The assumption of more complex relations, which doubtless are better approximations, leads to values of the median dose that differ from those calculated here by amounts so small as to be of no significance in this study.

\* The 5,5-disubstituted derivatives of another compound related to barbituric acid, oxazolidinedione, are reported by Stoughton and Baxter (8) to have no lag.

chemical groups, no others have been found to have lags.<sup>6</sup> In table 4 are listed a few of the anesthetics that have immediate onset of action. This list is chosen to contain representatives of diverse chemical classes and to show a wide range of anesthetic doses.

Examination of table 1 suggests that among the 5,5-disubstituted barbituric acids there is a tendency for the longer lags to be found in the less active drugs. That this association is statistically significant can be demonstrated by the treatment of figure 2. Here are plotted the anesthetic doses and lags of the 18

TABLE 1

*Anesthetic doses and lags of eighteen 5,5-disubstituted derivatives of barbituric acid*

The tabulated lag is the mean time of onset of anesthesia in 25 mice given a dose  $1.25 \times$  the AD 50.

BARTITURIC ACID	MOL. WT.	AD 50		MEAN LAG IN ONSET
		mgm. per gram	moles $\times$ $10^{-3}$ per gram	
5-ethyl-5-ethyl (Barbital).....	184.1	0.234	127	22.0
-5-phenyl (Phenobarbital).....	232.1	0.134	58	12.3
-5-isopropyl (Ipral).....	193.1	0.110	56	9.2
-5-(1-cyclohexenyl) (Phanodorn).....	236.1	0.110	47	0.9
-5-butyl (Neonal).....	212.1	0.076	36	1.6
-5-hexyl (Ortal).....	240.2	0.078	32	0
-5-isoamyl (Amytal).....	226.2	0.054	24	0.2
-5-(1-methyl-1-but enyl) (Delvinal).....	224.1	0.047	21	1.7
-5-(1-methylbutyl) (Pentobarbital).....	226.2	0.033	15	0.1
5-allyl-5-allyl (Dial).....	208.1	0.073	35	12.8
-5-isopropyl (Alurate).....	210.1	0.058	28	12.4
-5-isobutyl (Sandoptal).....	224.1	0.063	28	3.4
-5-(2-cyclopentenyl) (Cyclopal).....	234.1	0.060	26	1.6
-5-(1-methylbutyl) (Seconal).....	238.2	0.028	12	0.1
5-(2-bromoallyl)-5-isopropyl (Nostal).....	289.0	0.071	25	9.5
-5-sec-butyl (Pernoston).....	303.1	0.049	16	2.7
-5-(1-methylbutyl) (Sigmodal).....	317.1	0.040	13	0.2
5-methyl-5-(1-cyclohexenyl).....	222.1	0.206	93	8.9

barbituric acids of table 1. Lines are drawn through the mean values of dose and lag, dividing the plot into 4 quadrants. If anesthetic dose and lag were independent properties, the ratio of the number of drugs falling in Quadrant II to the number in Quadrant III should equal the ratio of the number in

<sup>6</sup> So far as I am aware, the only other drug that has been reported to have slow onset of action after intravenous administration is 3,3-diethyl-2,4-diketo-piperidine (itself similar in structure to barbital). Foster (9) found a lag with this drug in rabbits. In rabbits I also find a brief lag, but not in mice.

Quadrant I to the number in Quadrant IV. The observed ratios differ significantly, indicating that anesthetic dose and lag are associated properties.<sup>7</sup>

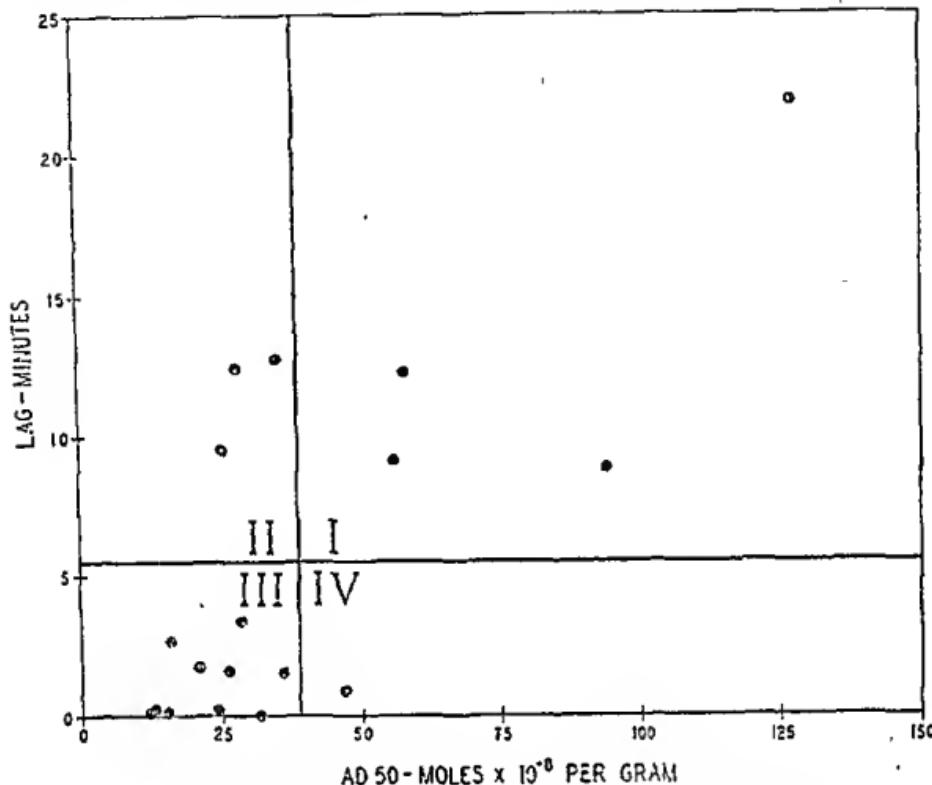


FIG. 2. THE EIGHTEEN 5,5-DISUBSTITUTED BARBITURIC ACIDS OF TABLE I  
The perpendicular lines are drawn through the mean values of dose and lag

No *N*-substituted barbituric acid or thiobarbituric acid was found that had a lag (table 2). Although a *N*-substituted compound is more active than the

<sup>7</sup> Of the 18 drugs, 7 have lags above the mean, 11 below the mean. If dose and lag were independent properties, the 13 drugs having anesthetic doses below the mean should contain the same proportion of high lags and low lags as 13 drugs selected at random from the 18. If 13 drugs are selected at random from the 18, the probability that these 13 will contain 10 or more below-the-mean lags is

$$\frac{5! 13!}{18!} \left( \frac{7! 11!}{4! 3!} + \frac{7!}{5! 2!} \right) = 0.047$$

This probability is so low that the hypothesis of independence cannot reasonably be accepted.

This test of independence involves no assumptions regarding the form of distribution of lags or doses, nor regarding the form of the relationship between dose and lag. Because the distributions of the doses and the lags cannot be regarded as normal (the distribution of these 18 doses shows significant positive skewness), the correlation coefficient cannot be applied here.

corresponding unsubstituted compound and a thiobarbituric acid more active than its oxygen analogue, it is to be noted that some of the drugs in table 2 are less active than the rapidly acting compounds of table 1.

DISCUSSION. The earliest attempt to explain the slow onset of action of a narcotic is that of Overton (10) (1901). He found that the narcosis produced by chloralose develops slowly in tadpoles and disappears slowly when they are transferred to fresh water. Chloralose was the only strong indifferent narcotic known by Overton to behave in this manner. By plasmolytic experiments, a method entirely independent of the appearance of narcosis, he demonstrated that chloralose enters plant cells slowly. In this respect, also, it differed from the other indifferent narcotics. Overton thought that the rapidity of entrance of a compound into a cell is determined by its solubility in the lipoids of the cell

TABLE 2

*1,5,5-trisubstituted derivatives of barbituric acid and 5,5-disubstituted derivatives of thiobarbituric acid*

The onset of action of all of these drugs is almost immediate.

BARBITURIC ACID	MOL. WT.	AD 50	
		mgm. per gram	moles $\times 10^{-8}$ per gram
1-methyl-barbital.....	198.1	0.070	35
1-ethyl-barbital.....	212.1	0.110	52
1-propyl-barbital.....	226.2	0.038	17
1-allyl-barbital.....	224.1	0.055	25
1-butyl-barbital.....	240.2	0.060	25
1-methyl-pbenobarbital (Mebaral).....	246.1	0.050	20
1-methyl-alurate (Narcenunal).....	224.1	0.032	14
1,5-dimethyl-5-(1-cyclohexenyl)-barbituric acid (Evipal).....	236.1	0.029	12
5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid (Pentothal).....	242.2	0.012	5
5,5-diethyl-2-thiobarbituric acid.....	200.2	0.140	70

membrane relative to its solubility in water. He evidently assumed that narcosis is produced only after the penetration of the drug into the cell.

Of a different nature is the suggestion offered by Klimesch (5) to account for the slowly developing action of certain barbituric acids. Klimesch attributed the differences in rapidity of onset of action of barbituric acids to the differences in degree of hydrolysis of their sodium salts. The greater the proportion of the drug present in the blood and tissues in the form of the lipid-soluble acid, the more rapid the onset of action was assumed to be. This idea was reaffirmed in a subsequent paper by Starkenstein and Klimesch (11). Bush (12) has pointed out that the method used by Klimesch to estimate the degree of hydrolysis of the salts is not valid. Bush determined the dissociation constants of five barbituric acids and, failing to find these values correlated with the lags, showed that the hypothesis of Klimesch is not acceptable. Barbital and amyta, two drugs that have lags differing in the extreme, were found to have equal

dissociation constants. The independence of dissociation constant and lag is corroborated by the study of a longer list of dissociation constants recently published by Clowes *et al.* (13). Fourteen of the barbituric acids of table 1 are to be found in this list. Among these fourteen, there is no evidence of association between  $pK'$  and lag.\*

Owing to the infinite complexity of the cell, the possibilities of describing its functions in terms of familiar physico-chemical models are severely limited. However, since the greater part of the time must be accounted for by processes the course of which is greatly affected by small changes in chemical structure or physical properties, it is possible that a study of the association between lag and the properties of the drugs may yield a clue at least to the general nature of the principal factor in the delay.

The first question that arises concerning the lag is whether it is the time required for the drug to arrive at its site of action or whether it is a delay in the biological response of the cell after the primary action of the drug is complete. Among the 5,5-disubstituted barbituric acids listed in table 1, drugs that differ among themselves relatively little in chemical structure, there are to be found lags of all magnitudes. It is difficult to conceive of drugs so closely related chemically differing among themselves in their sites or mechanisms of action. On the other hand, the differences in physical properties are such as might be expected greatly to influence the speed of passage from the blood to the site of action. Therefore, at least so far as the barbituric acids are concerned, the hypothesis that the delay is in the arrival of the drug at its site of action appears the more attractive.

The significant association between narcotic activity and rapidity of onset demonstrated here may throw some light on the nature of the process by which the drug reaches its site of action. Such an association would result if this mechanism were dependent on some physical property of the drug that is also associated with narcotic activity. Among the barbituric acids as well as other narcotics, there is a general tendency for the more active drugs to have a higher solubility in certain organic solvents relative to water. This solubility relationship is often thought of in terms of the distribution coefficient between olive oil and water, not because of any unique physiological significance of this particular coefficient but because it was the one used by the early advocates of the lipid theory of narcosis. Let it be assumed that the lag in onset of narcosis is due to diffusion of the drug from an aqueous phase into or across a non-aqueous phase. Then if the more active drugs had higher partition coefficients between the non-aqueous solvent and water, they would pass more rapidly into and across the non-aqueous phase, and the observed association between anesthetic dose and lag would result.

The fact that there is also a marked parallelism between the oil/water distri-

\* If these data are treated by the method of figure 2,  $pK'$  (from Clowes *et al.*) being plotted as abscissa and lag (from table 1) as ordinate, the number of drugs falling in quadrants I, II, III, and IV are, respectively, 3, 3, 4, 4. These numbers do not differ from those expected from the hypothesis of independence.

bution coefficients of many organic substances and their speeds of penetration through cell membranes suggests that the non-aqueous phase assumed above might be the lipoid layer of the cell membrane.<sup>9</sup> If some of the assumptions considered above were combined, the hypothesis would assume this more specific form:

The delay in onset of narcotic action is the time required for the drug to penetrate into or through the cell membrane, the more rapid penetration of the more active drugs being due to their greater relative solubility in the lipoids of the membrane.

TABLE 3

*Anesthetics other than barbituric acids having slow onset of action*

The lag was measured in the same way as for the barbituric acids of table 1.

DRUG	MOL. WT.	AD 50		MEAN LAG IN MINUTES
		mgm. per gram	moles $\times$ $10^{-4}$ per gram	
5-ethyl-5-phenyl-hydantoin (Nirvanol).....	204.1	0.161	79	14.5
$\alpha$ -d-glucocchloralose.....	309.5	0.082	27	24.4
$\alpha$ -d-arabinocchloralose.....	279.4	0.410	147	15.6
$\alpha$ -l-arabinocchloralose.....	279.4	0.199	71	9.8

TABLE 4

*Some anesthetics other than barbituric acids having immediate onset of action*

DRUG	MOL. WT.	AD 50	
		mgm. per gram	moles $\times$ $10^{-4}$ per gram
Methanol.....	32.0	7.0	22,000
Chloral hydrate.....	165.4	0.27	163
Ethyl acetate.....	88.1	0.27	306
Ethyl carbamate.....	89.1	1.12	1,250
Paraldehyde.....	132.1	0.73	553
Acetone.....	58.0	2.4	4,140
Sulfonal.....	228.3	0.50	219

This hypothesis is essentially an application of Overton's theory of cellular permeability. However, it is to be noted that it has been assumed above that the amount of drug passing from the original aqueous phase is at least roughly proportional to the observed anesthetic dose. This assumption is not consistent with the requirements of the lipoid theory of narcosis, at least as set forth by Meyer and Hemmi (15).

The hypothesis mentioned above is inadequate to explain the unequal rates of onset of narcosis following injection of the enantiomeric arabinocchloraloses,

<sup>9</sup> Among the 9 barbituric acids of table 1 for which olive oil/water distribution coefficients have been published (14) there is a significant association between distribution coefficient and lag.

for the physical properties of these compounds are identical. It is conceivable that the chloraloses differ entirely from the barbituric acids in their site and mechanism of action. The lag might still be considered a delay in the arrival of the drug at its site of action if it were assumed that the drug penetrates not through simple diffusion in accordance with Fick's law, but by some process that limits the rate of transfer. Thus the larger quantity of a less active drug would require a longer time to reach the site of action.

No reason is apparent why the lag should be found in such a limited number of chemical classes of narcotics. Reference to table 4 indicates that it cannot be solely a matter of narcotic activity or of molecular dimensions or of solubility. One must assume that those chemical groups in which lags are found narcotize at a different site or through a different mechanism or arrive at their site of action through a different mechanism from other narcotics.

#### SUMMARY

A delay occurs between the intravenous introduction of certain anesthetics and the full development of their action. The only drugs that I have found to have this property are chloraloses and 5,5-disubstituted derivatives of barbituric acid and hydantoin. The lags of a number of these drugs have been measured at doses designed to give comparable depths of anesthesia.

Among the eighteen 5,5-disubstituted barbituric acids studied, it has been demonstrated that anesthetic dose and lag are associated properties, the more active drugs tending to have more rapid onset of action.

This association might be explained if it were assumed that the delay is the time required for the drug to penetrate into or through the cell membrane, the more rapid penetration of the more active drugs being due to their greater relative solubility in the lipoids of the membrane.

This hypothesis is inadequate to explain the unequal rates of onset of the antipodal arabinochloraloses.

No reason is apparent for the fact that the property of slow onset of action is limited to those chemical classes named.

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## SOME PROPERTIES OF FICIN

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During recent years a number of investigators have reported studies on the properties of the enzyme contained in the milky sap of the fig. Such studies have been prompted by its enzymatic properties as well as by its historic status as a Spanish-American antihelmintic. An excellent review of its history and bibliography is given by Asenjo (1). The purpose of this paper is to present the results of further investigation along lines suggested by its use as an antihelmintic.<sup>1</sup>

The partially purified enzyme, dried to constant weight in a vacuum desiccator at room temperature showed, by Kjeldahl, a total nitrogen content of 11.1 per cent, corresponding to 69.6 per cent protein. Its average ratio of free amino nitrogen to total nitrogen, the former determined by the Van Slyke method, ranged from 13 to 14 per cent in different samples, which places it approximately in the proteose class. In its proteolytic properties it resembles papain in that it has an optimum pH of about 5, as shown by Robbins (2). This pH was therefore used for all determinations of proteolytic activity. We found that it rapidly hydrolyzed proteins such as egg albumin, gelatin, etc. at room temperature, but carried them to the proteose-peptone stage with a free amino nitrogen total nitrogen ratio of about 25 per cent. When added to commercial proteose-peptone mixtures little or no further hydrolysis was observed. Thus in the degree of hydrolysis accomplished it resembles gastric pepsin.

The thermo-lability of ficin was first reported by Robbins (3), who used its effect on *Ascaris* as the criterion. We have confirmed his results using hydrolysis of egg albumin, measured by the production of non-precipitable (by trichloroacetic acid) nitrogen as the criterion. Complete inactivation resulted from one hour's heating at from 70° to 75°C. The enzyme is not poisoned in any measurable degree by the presence of chloroform. Treatment of a water solution with ketene under conditions which should acetylate only free amino groups yielded a product with about two-thirds the enzymatic activity of the untreated sample.

The fairly homogeneous character of our enzyme preparation is indicated by the results of dialysis through a collodion membrane. As is the case with most proteose-peptone mixtures, a portion dialyzes easily through such a membrane but the molecular complexity of the dialysate, as indicated by the free amino

<sup>1</sup> The work was carried on with partially purified samples of the enzyme in powdered form furnished us by the kindness of Merck and Company, Incorporated.

nitrogen total nitrogen ratio, does not differ markedly from that of the residue. Furthermore, experiments of this type have shown enzymic activity on the part of the dialysate proportional to the total protein content of the solution.

One of the remarkable properties of this enzyme, no doubt responsible for its continued medicinal use, is that of digesting living intestinal helminths. Such ability obviously does not accord with our conception of the mechanism of action of proteolytic enzymes but a member of experiments with freshly collected, live, pig *Ascaris* provide ample confirmation of the results first reported by Robbins (3). The helminths are rapidly attacked by the enzyme with the production of extensive lesions of the body wall. During the course of this digestion the power of locomotion of the worms leaves no doubt that they remain alive for some time. However, long-continued action by the enzyme solution produces death and almost complete disintegration of the worms, with marked increase in the nitrogen content of the solution in which they are immersed.

*Site of action.* In an attempt to determine whether the action of ficin on live *Ascaris* is on the exterior body surface or through the alimentary tract of the worms, the following experiment was carried out: Freshly collected live *Ascaris* were divided into three groups of five worms each. *Group I* was immersed in 200 cc. 0.1 per cent ficin made in Locke's solution; *Group II* was immersed in the same volume of Loeke's solution without the ficin. *Group III* was a duplicate of *Group I* in the ficin solution but, whereas the worms of *Group I* had their alimentary tract closed by ligation as described by Brown (4), those of *Group III* were left unligated. All three groups were incubated at 37°C. and observed at intervals up to 150 minutes. At the end of that period the worms of *Groups I* and *III*, while retaining the power of locomotion, had a badly blistered cuticular surface whereas *Group II* (controls) showed no damage. However, no difference could be observed between *Groups I* and *III*; since ligation of the alimentary tract was without result, the action of the enzyme was purely on the exterior surface of the worms. Moreover, the increase in the nitrogen content of the solution as digestion proceeded was practically identical in *Groups I* and *III* in spite of the inability of the enzyme to gain entrance to the intestinal tract of the worms of *Group I*. A similar experiment in physiological saline solution gave the same results.

*Fecal excretion.* The clinical use of crude preparations of ficin for the treatment of *Trichuriasis* has been described by Brown (5), and recommended by various writers as reviewed by Asenjo (1). The degree of success recorded for crude ficin preparations is the more remarkable because the habitat of *Trichuris* is the cecum. Thus the necessary site of action is so far down the intestinal tract as to subject the enzyme, evidently of protein nature, to the digestive action of the proteolytic enzymes of the stomach and small intestine. The literature contains little or no exact information as to the relative abilities of different proteolytic enzymes to destroy one another but one must assume, in this case, that some of the ficin passes unaffected through the areas of tryptic and creptic digestion and arrives at the cecum with, at least its anthelmintic if not its proteolytic properties unimpaired. In this ability, it appears to be in sharp contrast to the

animal's ordinary digestive enzymes which are almost entirely absent from normal feces. It seemed worth while, therefore, to test whether or not the proteolytic activity of doses of ficin can survive to the point of excretion in the feces. It is well known that feces normally show little or no tryptic activity.

The question of fecal survival of ficin was tested as follows: Doses of partially purified and dried ficin (used in the above-described experiments) were administered in water solution to two dogs at levels of 0.1 gram and 0.5 gram respectively per kilo body weight. Twenty-four hour collections of feces were made, both for several days before administration of the enzyme and for a longer period afterwards. The dogs were kept throughout the experiments on an ordinary animal house diet. The 24-hour samples of feces, varying considerably in weight, were each macerated in water; the resulting mixture was filtered and washed into 500 cc. volumetric flasks and made up to volume; 25 cc. aliquots of these solutions were used for testing proteolytic activity.

Standardization was effected by running a series of tests with varying amounts of the same sample of ficin as that administered to the dogs in 25 cc. water. This was added to

TABLE 1

*Absolute increase in percentage of free amino- to total nitrogen effected by varying amounts of ficin in 25 cc. water solution added to 200 cc. 2% gelatin solution. Room temperature*

WEIGHT OF FICIN IN 25 CC. SAMPLE	ABSOLUTE RISE IN PERCENTAGE OF FREE AMINO- TO TOTAL NITROGEN	
	30 minutes <sup>1</sup>	60 minutes
GRAMS		
0	0	0
0.05	3.25	3.90
0.10	5.51	6.68
0.15	6.68	7.84
0.20	7.95	8.96
0.25	8.69	9.64
0.30	9.28	10.64

200 cc. 2% gelatin solution at room temperature and the resulting ratio of free amino- to total nitrogen determined at once, after 30 minutes, and after one hour. The amounts of ficin in the 25 cc. samples were varied from zero to 0.30 gram but the amounts present in the 25 cc. aliquots of the feces solution never exceeded the equivalent of 0.05 gram. A straight-line relationship was assumed to hold sufficiently well between 0 and 0.05 gram to permit interpolation. The original ratio in the gelatin was 2.92%. The results of the standardization are recorded in table 1, the absolute rise in the ratio for 30 and 60 minutes over the ratio as determined at once being recorded in relation to the amounts of ficin contained in the 25 cc. sample of the same.

The enzyme content of the 24-hour samples of feces was determined by direct proportion; the data used were those given in table 1 for a hydrolysis period of one hour. The 25 cc. aliquot of the feces solution was mixed with 200 cc. 2% gelatin solution. This solution plus 25 cc. H<sub>2</sub>O had, as stated above, a ratio of free amino- to total nitrogen of 2.92%. After one hour hydrolysis at room temperature the ratio was determined and corrected for the nitrogen introduced into the feces solution. From this ratio there was subtracted the original ratio determined on the gelatin (2.92%). To apply a further correction for the small enzymatic activity encountered in normal feces, an average was made of the values similarly obtained on several control periods when no ficin had been administered. This average amounted to 0.75% absolute increase under identical conditions (see table 2) for

nitrogen total nitrogen ratio

Furthermore, experiments of  
of the dialysate proportional to

One of the remarkable properties  
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ability obviously does not accord with  
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pig Ascaris provide ample confirmation.

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*Site of action.* In an attempt to determine  
live ascaris is on the exterior body surface or through  
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were divided into three groups of five worms each.  
200 cc. 0.1 per cent ficin made in Locke's solution; Group I  
same volume of Locke's solution without the ficin. Group II  
of Group I in the ficin solution but, whereas the worms  
alimentary tract closed by ligation as described by Brown (4),  
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ment with a sample of papain paste<sup>2</sup> produced similar results, the recoveries having reached a maximum of about 63 per cent of that administered.

It is, of course, recognized that such a heavy dose of a highly irritating enzyme (cf. 6) produces abnormal conditions in the intestine. In the above-described experiments with ficin the resulting feces were patently abnormal, being soft and quite red in color, undoubtedly indicating some bleeding. However the animals quickly recovered their normal condition.

#### CONCLUSIONS

Further investigation of the properties of the fig enzyme (ficin) have shown that:

1. The action on *Ascaris lumbricoides* is on the exterior of the live worm and not through its alimentary tract.
2. The similar effect on *Trichuris* in the cecum is explained by its ability to pass through the digestive tract of the dog and to be excreted in the feces with a large share of its proteolytic activity intact.

We wish to acknowledge the assistance of The Samuel S. Fels Fund for the means to carry on this investigation.

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# INTRAVENOUS INJECTIONS OF SOLUBLE BISMUTH COMPOUNDS: THEIR TOXICITY, AND THEIR SOJOURN IN THE BLOOD AND ORGANS<sup>1</sup>

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Intravenous injection of water soluble bismuth compounds is not used clinically because of the relatively high toxicity, which had been shown experimentally and confirmed in the few clinical trials that have been reported.

H. Beerman, 1932 (1), found reports of five *acute deaths of human patients* from intravenous injection of bismuth compounds. The following abstract is typical of such occurrences: S. H. Curtis, 1930 (2), had used intravenous injections of a solution of "bismuth tartrate" (presumably sodium bismuth tartrate) in ten patients, without any reactions. He injected the equivalent of 15 mgm. of bismuth (approximately 0.25 mgm. per kilogram of body weight) in 5 cc. of sterile distilled water, weekly for ten doses. An eleventh patient, however, a young man in good physical condition, reacted to each of the ten injections with abdominal pain and one or two watery stools. A month later another course was started, but the patient died within five minutes of the first injection, with stormy symptoms of colloidoclastic shock, cyanosis, collapse and immediate loss of consciousness.

The introduction of the slow intravenous drip method of administering relatively large doses of neoarsphenamine and mapharsen (3), suggested to Dr. H. N. Cole that similarly slow injection of bismuth salts might decrease their toxicity sufficiently so that they could be utilized, especially as the experimental fatalities from intravenous bismuth administration often occur suddenly during or very shortly after the injection, suggesting flocculation, which could perhaps be avoided by slow intravenous injection. In any case the phenomena of intravenous bismuth injection called for further study, especially in comparison with the central nervous symptoms that are so prominent in poisoning by alkyl bismuth (4), and which are absent in ordinary bismuth poisoning. The difference could conceivably be due to the more rapid absorption of the alkyl compounds. It also appeared desirable to make quantitative studies of organ distribution and of blood concentration of bismuth when the dosage entering the blood could be absolutely controlled. For comparison, studies were made with single injections in ten to thirty minutes ("fast injection"), and in four to ten hours ("slow

<sup>1</sup> This investigation was supported by a grant of the John and Mary R. Markle Foundation. A preliminary communication was presented to the American Society for Pharmacology and Experimental Therapeutics at its meeting of April 15-19, 1941, in Chicago.

injection"), and with injections, slow or fast, repeated daily up to five days. The studies were begun on unanesthetized rabbits, and extended to dogs, without and with anesthesia.

*Method for prolonged intravenous injection of unanesthetized rabbits.*—The animal is placed in a rabbit injection box like that described by D. E. Jackson, but with several large holes bored through the sides and bottom for ventilation. The head protruded through the V-shaped cut and was freely movable. The box was placed in a trough tilted to drain urine through the bottom holes. After the hypodermic needle (usually gauge 22 or 24) had been inserted into the marginal ear vein and clamped to the ear by means of an artery clamp, the ear was fastened to the lid. This arrangement served at least twelve hours, if the needle was nearly parallel to the vein, and the flow of solution was not too slow. The solutions for slow injections were prepared by dissolving the calculated daily amount of the bismuth compound together with 12.5 grams of dextrose in 250 cc. of distilled water. The mixture was placed in a liter flask which was painted with aluminum on the outside to retard the action of light. A Murphy drip tube, previously calibrated for size of drops, was then attached and the flow regulated by means of a pinch clamp. Under ordinary conditions the flow was 0.5 cc. (about eight drops) per minute, the total injection lasting about eight hours. Three types of water soluble bismuth compounds were used, as represented by sodium bismuth citrate, sodium bismuth thioglycollate (thiobiomol) and sobisminol.<sup>3</sup> The dilute solutions of these compounds flocculate slowly on standing, first the thioglycollate, then the citrate, and last the sobisminol. Under the conditions of our experiments a Tyndall effect was observable in one to four, six to eight and eight to twelve hours, visible flocculation in four to six, eight to sixteen, and sixteen hours, respectively. None of the fresh solutions produced visible turbidity in dog or rabbit serum in less than two hours when added *in vitro*.

**TOXIC EFFECTS IN RABBITS.** The outcome of the injections may be grouped as acute fatality, delayed death, and indefinite survival. The *acute deaths* include the animals that died during or shortly after an injection. The symptoms were typically those of flocculation shock. Death was preceded by dyspnea, salivation and asphyxial convulsions. Autopsy showed pulmonary emphysema ("butterfly lung"). The *delayed deaths* occurred in 12 hours to 27 days, the time generally varying inversely with the dose. Death was due to nephritis. Extensive edema was present in the animals. No other signs were observed, except that the characteristic black deposits of bismuth occurred in the cecum. The *indefinite survivors* were killed 30 to 57 days after injection. The animals in this group did not show any signs of toxicity during life, and nephritic changes when present were of a much less degree than in the delayed deaths.

The nephritis and edema are evidently related to the severity of the intoxication and the time of survival. Edema was observable in about 20 per cent of the acute, 30 per cent of the subacute, 65 per cent of the delayed, 10 per cent of the late fatalities, and in none of the animals that survived indefinitely. Severe nephritis was present in all the fatalities. It was much more moderate in the

<sup>3</sup> *Sobisminol Solution, N.N.R.*, contains "a complex organic bismuth product the chemical nature of which has not been fully established. It is obtained by dissolving the products of the interaction of sodium bismuthate, triisopropanolamine and propylene glycol in a mixture of propylene glycol and water. Each cubic centimeter of the solution contains between 19.5 and 20.5 mgm. of bismuth and 0.5 cc. of propylene glycol."

animals that survived and were sacrificed after four weeks; in some of these no nephritis was noticeable on gross examination.

*Fatal doses.*—Table 1 presents the fate of the rabbits with *single fast* injections of the various bismuth compounds, arranged in descending order of dosage. They show that the LD<sub>50</sub> (the dosage that is lethal for two thirds of the animals), when injected intravenously into rabbits in about ten minutes, corresponds approximately to 4 mgm. of Bi per kilogram of body weight with sobisminol, to 2.5 mgm. with the citrate, and to 3.5 mgm. with the thioglycollate. Excluding the acute fatalities from these data does not change the LD<sub>50</sub>. The difference in the LD<sub>50</sub> of these compounds, as related to fast intravenous injection, is not large and may fall within the limit of statistical error. Our figure for sobisminol agrees closely with those given by Hanzlik, Lehman and Richardson (5), viz. 4.2 for LD<sub>50</sub>, 6.3 for LD<sub>100</sub>; that for sodium bismuth citrate approximates that of Longley, Clausen and Tatum (6). With thioglycollate, Gruhzeit, Lyons and Perkins (7), reported an LD<sub>100</sub> of 8 to 10 mgm., which is almost three times our LD<sub>50</sub> figure.

Table 2 presents the toxicity of "*slow injections*." With these, the LD<sub>50</sub> is 10.5 mgm. with sobisminol, 5.0 with the citrate, 2.8 with the thioglycollate. Comparison of the fast and slow injections shows that with the latter method the toxicity of all is reduced approximately by one half, excepting the thioglycollate, which appears actually more toxic with slow than with fast injections. This is due to the high incidence of acute fatalities during or at the end of the injection, and is perhaps caused by a physical change of the labile thioglycollate during the slow injection, or during the prolonged sojourn in the blood-stream which was shown by studies of its blood concentration in dogs; these data will be presented later in the paper.

Table 3 shows the fate of the rabbits with *repeated slow injections*, the aim being to administer five equal fractions on successive days, by the technic described for single slow injections. The results appear too irregular to assign a definite L.D. The complication is due chiefly to the incidence of fatalities during or shortly after relatively small final doses. Our impression is that spreading the dosage over five days decreases the *late* toxicity somewhat, but not to a practically important extent, and that it increases the liability to acutely fatal reactions in a very dangerous degree.

*Incidence of acute deaths.*—Animals that die during or within a few minutes after the injection show pulmonary distention and other phenomena of flocculation shock, differing materially from the fatalities that occur later. They evidently belong to a different category. In a sense they are "accidental," involving some peculiar factor which is not clearly related to the dosage. The fatalities with single fast injections occur with relatively small doses (3.5 and 2.5 mgm. for sobisminol, 5 mgm. for the citrate), and at a considerably higher dosage with slow single injections (14.2 and 38 mgm. for sobisminol; 15 mgm. for citrate; 34, 25, 18, 4 and 2.8 for thioglycollate). The incidence (the ratio of acute deaths to all injections) is about twice as great for fast as for slow single injections with sobisminol (fast, 2:9; slow, 2:20) and citrate (fast 1:9; slow

1:16). Thioglycollate shows a peculiar behavior, since none of the six rabbits with fast injections died, but 5 out of 9 did with slow injection. We suspect that this may be due to the instability of the thioglycollate solution during the

TABLE 1

*Outcome of single fast intravenous injection of bismuth compounds for rabbits*

The bismuth compound was dissolved in 5 cc. of 5 per cent dextrose and injected, generally at the rate of 0.5 cc. per minute, so that the total injection usually required 10 minutes.

DOSE AS M.M. Bi PER KGM.	SOBISMINOL	Na Bi CITRATE	Na Bi THIO- GLYCOL- LATE
9			+ 1 d.
7			+ 4 d.
5	+ 4 d.	+ 5 m.	
5	+ 5 d.	+ 5 d.	
3.5	+ end of inj.		+ 21 d.
3.5	K 34 d.		+ 2 d.
3.5	K 37 d.		
3.0		+ 6 d.	+ 9 d.
3.0		+ 8 d.	K 37 d.
3.0		+ 10 d.	K 41 d.
3.0			K 41 d.
2.5	+ during inj.	+ 11 d.	
2.5	K 25 d.	+ 14 d.	
2.5	K 43 d.	+ 33 d.	
2.0	K 35 d.		
LD <sub>50</sub> (approximate)	4 mgm.	2.5 mgm.	3.5 mgm.

Abbreviations: d = day; m. = min.; + = time of death; K = time of sacrifice; h = hour.

TABLE 2

*Outcome of single slow intravenous injections for rabbits*

Most of the injection volumes were 250 cc. in 8 hours; some 500-600 cc. in 10 to 14 hours.

DOSE AS M.M. Bi PER KGM.	SOBISMINOL	Na Bi CITRATE	Na Bi THIO- GLYCOLATE
34-40	+ dur. inj.		+ dur. inj.
	+ 1 d.		
25-28	+ 3 d.		+ dur. inj.
	+ 5 d.		
18-20	+ 3 d.	+ end of inj.	+ end of inj.
	+ 3 d.		
	+ 5 d.		
	+ dur. inj.		
12-15	+ 6 d.	+ 1 d.	+ 6 d.
	+ 8 d.	+ 3 d.	
	+ 8 d.		
	+ 10 d.		
10-11	+ 6 d.	+ 8 d.	
	+ 7 d.	+ 9 d.	
	+ 10 d.		
	+ 10 d.		
	K 33 d.		
	K 39 d.		
	K 58 d.		
	K 70 d.		
	8.0	+ 9 d.	
		K 50 d.	
7.0		+ 4 d.	
		+ 4 d.	
6.0		+ 2 d.	
		+ 8 d.	
		+ 10 d.	
6.0		+ 9 d.	
		+ 18 d.	
		K 57 d.	
4.0		+ end of inj.	
2.8		+ dur. inj.	
2.5		+ 14 d.	
		K 51 d.	
		K 51 d.	
LD <sub>50</sub> (approximate)	10.5 mgm.	5.0 mgm.	2.8 mgm.

long injection, although special care was taken to minimize this by protecting the solution against light and by renewing it at one to four hour intervals.

The incidence of acute deaths appears distinctly higher for repeated injections than for single injections, double in the case of sobisminol (3:14 repeated as

against 2:20 for single); four times for citrate (2:8 repeated, 1:16 single). For thioglycollate, however, the incidence of acute deaths was lower for repeated injections (1:4) than for single slow injections (5:9). The dosage at which acute death occurred is strikingly different for repeated and single slow injections. The amount of bismuth which was injected during the last of the series of repeated injections in which acute death occurred ranged between 0.7 to 2.5 mgm., median 1.6 mgm., while for single slow injections it ranged between 2.8 and 40 mgm., median 16 mgm. The median dose at which acute death occurred in

TABLE 3  
*Outcome of repeated (daily) slow intravenous injections for rabbits*

MOM. BI KGM. AT EACH INJ.	SOBISMINOL			Na Bi CITRATE			Na Bi THIOGLYCOLLATE		
	Number of doses	Total Bi		Doses	Total Bi		Doses	Total Bi	
16.7	16.7; 7.3	24	+ 2 d.						
12	12; 12	24	+ 2 d.						
3.8							3.8; 3.8; 3.8; 1.9	13.3	+ end inj.
2.5	4½	11.5	+ during						
	4½	10.8	K 38 d.						
	3½	8.75	+ 24 d.						
	3	7.5	+ end inj.						
	1½	4.25	+ 15 d.						
2.0	5	10	K 39 d.	5	10	+ end inj.			
	5	10	K 39 d.	5	10	K 42 d.			
	5	10	K 39 d.	5	10	K 42 d.			
1	2½	2.7	+ during	5	5	+ end inj.	5	5	+ 8 d.
1	5	5	K 31 d.	5	5	K 28 d.	5	5	K 34 d.
1	5	5	K 43 d.	5	5	K 41 d.	5	5	K 48 d.
1	5	5	K 48 d.						
0.5				5	2.5	+ 27 d.			
Fast				5	2.5	K 33 d.			
LD <sub>50</sub> —total bis- muth—mgm. per kgm. (ap- proximate)	8				7.5				

repeated injections is therefore only one tenth that for single slow injections. The proportion of such fatalities increased with the number of repeated injections, for of the twenty six rabbits injected repeatedly, none died acutely with the first or second injection, two died with the third, one with the fourth and three with the fifth injection. We are inclined to attribute the acute deaths from small doses after repeated injections to thrombosis and embolism, arising from trauma and inflammation of the delicate vein of the rabbit's ear. Flocculation between blood and the bismuth solution is not materially hastened by repeated injections, as was seen by layering the bismuth solutions over serum and also by mixing serum with the solution to obtain a bismuth concentration com-

parable to that during injection of intact animals. Flocculation was visible in 1½-2 hours and precipitation in 14-18 hours, alike in the serum of rabbits that had not been injected with bismuth, and of those that were killed 4-16 hours after receiving five slow intravenous injections of the various bismuth solutions.

*Time of death in relation to dosage.*—Figure 1 shows that the dosage has a marked effect on the time of survival, both with slow and with fast injections. The average dosage of bismuth that kills rabbits in one day is 18 mgm. per kilogram, with slow injection; half of this dosage requires an average of nine days to kill. With fast injection, an average of 9 mgm. is fatal in one day and half of this kills in five days. The curves are somewhat parabolic; they are similar for the three compounds and for fast and slow injections; the few data

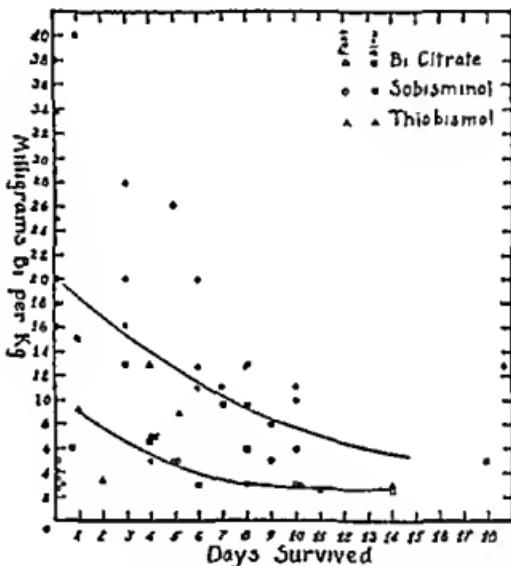


FIG. 1. RELATION OF DOSAGE TO SURVIVAL TIME, WITH INTRAVENOUS INJECTIONS OF BISMUTH SALTS

on repeated injections also run in the same direction. Rabbits that survive ten days generally recover.

*Comparison of bismuth toxicity with intramuscular injections of watery solutions.*—Even the slow intravenous injections are considerably more lethal to rabbits than intramuscular injections, for according to the data recorded in the literature the intravenous fatal dose is probably of the order of one fifth to one tenth that of the intramuscular injections.

More accurate comparisons are not feasible since the published data involve different criteria of survival and different periods of observation. For sobisminal, whose median  $LD_{50}$  we found to be 10.5 mgm. per kilogram by slow intravenous injection, Hanzlik, Lehman and Richardson (5), found a  $LD_{50}$  of 77 mgm. per kilogram for intramuscular injection, a ratio 1:7. For sodium bismuth citrate we found 5 mgm. for slow intravenous injection. Longley, Clausen and Tatum (6) give 10 mgm. as a "tolerated" intramuscular dose, a ratio

of 1:2. Leonard (8) reports 200 to 300 mgm. as the LD<sub>100</sub>, a ratio of 1:40. For sodium bismuth thioglycollate we found 2.8 mgm. for slow intravenous injection; Gruhzit, Lyons and Perkins (7) give the LD<sub>100</sub> as 10 to 12 mgm., a ratio of 1:4.

**TOXIC EFFECTS IN DOGS.** *Intravenous toxicity for unanesthetized dogs.*—The animals were trained daily for a week by loose strapping with elastic bandage to an animal board, and by fixed extension of the leg in which injections were to be made. Following the training period there was no serious difficulty in injecting the bismuth in 500 cc. of 5 per cent dextrose solution into the pedalic vein over periods of 4½ to 8 hours. Sobisminol was administered to all animals excepting one, which received the citrate. Three dogs that received a *single slow intravenous injection* of sobisminol in doses corresponding to 11, 7, and 7 mgm. bismuth per kilogram died in 3, 5 and 5 days respectively. Two injected with 5 mgm. of Bi as sobisminol and one with 3 mgm. as citrate survived indefinitely and were killed in 90, 36 and 60 days respectively. The LD<sub>50</sub> lay between 7 and 5 mgm. per kilogram, i.e., about half the LD<sub>50</sub> of rabbits given slow intravenous injections (10.5 mgm.). Dogs appear therefore somewhat more susceptible to intravenous bismuth poisoning than are rabbits, in agreement with the observations of Levaditi (9) and Brabant (10), but the difference is not beyond the range of statistical error. *Repeated slow injections* were made in two unanesthetized dogs each receiving five injections of sobisminol on successive days; one dog was given 2 mgm., the other 1 mgm. of Bi in each injection, making the total dose 10 and 5 mgm. respectively. Both survived until they were killed, after 38 and 34 days respectively. Although the data are few they indicate that perhaps a somewhat higher total dosage may be tolerated if it is spread over several days. The *symptoms* in delayed poisoning in dogs as in rabbits appeared to depend solely on the degree of kidney damage, and this in turn on the dosage of bismuth. Fatal cases developed the lethargy of uremia. If the dose was less than fatal, the animals remained in apparent good health during the observation period. When they were sacrificed the autopsy revealed varying degrees of kidney damage. There were no indications of bloody diarrhea, peripheral neuritis or encephalopathy.

*Toxicity of intravenous injections in anesthetized dogs.*—This was observed incidental to the study of the rate of disappearance of bismuth from the blood. The dogs were under barbiturate anesthesia (sodium isopropyl ethyl barbiturate 75 mgm. kgm. or sodium isopropyl allyl barbiturate 50 mgm. kgm.). The slow injections were made as in the unanesthetized dogs. Death occurred acutely at the end of the injection in the three dogs that received sobisminol in ten minutes; the dose of Bi was 2 mgm. per kilogram in one dog, 4 mgm. per kilogram in the others. The other 12 dogs survived at least 4½ hours after the end of the injection of 4 mgm. per kilogram of bismuth; two of these dogs were injected with sodium bismuth thioglycollate in ten minutes, two with sobisminol in twenty minutes, two with sobisminol and six with thioglycollate for 4½ hours. One of the latter died 4½ hours and one 36 hours after the end of the injection. It would appear that sobisminol is especially liable to produce acute death in anesthetized dogs if it is injected rapidly, i.e., within ten minutes, and that sub-

acute death may occur even with slow injection of the thioglycollate in the dose of 4 mgm. of Bi per kilogram which did not cause death in any of the nonanesthetized dogs. The explanation of the apparent potentiating effect of anesthesia on the acute and subacute bismuth toxicity is not clear.

*Sojourn of bismuth in the blood.*—The difference in therapeutic and toxic effects of slow and fast intravenous injections of bismuth compounds would depend chiefly on the level of concentration that is attained and maintained in the

TABLE 4

*Bismuth concentration in blood of dogs (mgm. Bi per 100 cc. of whole blood) after intravenous injection of soluble bismuth salts, in the ratio of 4 mgm. Bi per kgm. of body weight.*

Barbiturate anesthesia

Dog number	Body weight	Compound	Time (minutes) after end of injection:					24 HOURS	PER CENT OF BISMUTH WHICH HAS LEFT BLOOD STREAM BY END OF INJECTION	
			0	10	20	90	270			
			kgm.							
2	17.0	Thiob.	0.482	0.282		0.111	0.105		89	
6	17.7	Thiob.	0.355	0.214	0.220	0.177	0.094		91	
11	24.7	Sobis.	1.16	0.64	0.355	0.200	0.120		73	
12	15.5	Sobis.	0.800	0.303	0.203	0.132	0.036		82	
<b>B. SLOW INJECTIONS (4½ TO 5½ HOURS)</b>										
			During injection			After injection				
			30 min.	90 min.	End	30 min.	90 min.	270 min.		
3	24.0	Sobis.	0.071	0.174	0.247	0.100	0.092			94
8	13.6	Sobis.	0.054	0.084	0.100	0.070	0.044	0.021		97
4	28.0	Thiob.	0.071	0.350	0.357	0.364	0.342			92
7	13.9	Thiob.	0.121	0.142	0.200	0.166	0.200	0.214		95
13	15.3	Thiob.	0.077	0.110	0.130	0.092	0.077	0.074		97
14	17.5	Thiob.	0.036	0.092	0.133	0.100	0.075	0.056		97
9	24.6	Thiob.								
		Plasma	0.069	0.383	0.440	0.114	0.300	0.182		87
		Cells	0.011	0.063	0.089	0.093	0.068	0.025		
10	24.6	Thiob.								
		Plasma	0.081	0.355	0.325	0.250	0.285	0.120	0.051	91
		Cells	0.040	0.032	0.076	0.098	0.068	0.051	0.051	

blood. This was studied on dogs under barbiturate anesthesia (same as above), by injecting 4 mgm. of bismuth per kilogram of body weight, either as sobisminol or as sodium bismuth thioglycollate, as fast injections in 10 minutes for thioglycollate, in 20 minutes for sobisminol; and as slow injections in 4½ to 5½ hours for both. Samples of blood representing 5 cc. per kilogram were withdrawn at the end of the injection, and at 10, 30, 90 and 270 minutes after the end of the injection; in slow injections samples were also taken at a half hour and 1½ hours during injection. The samples, about 100 cc. each, were analyzed by the method described below for rabbit organs. The results are shown in table 4 as milligrams

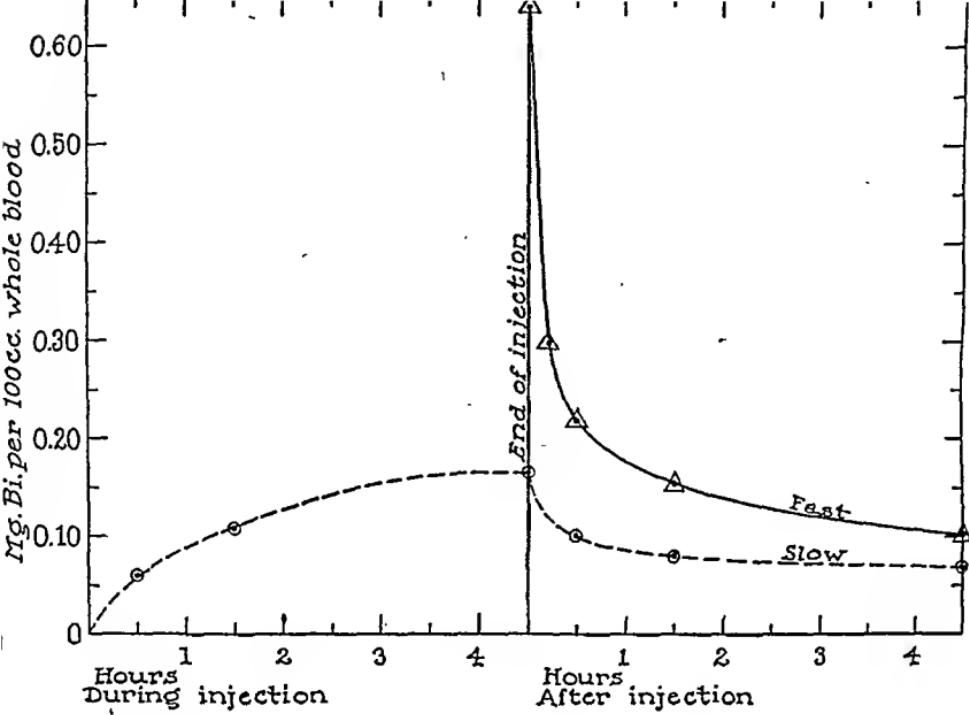


FIG. 2

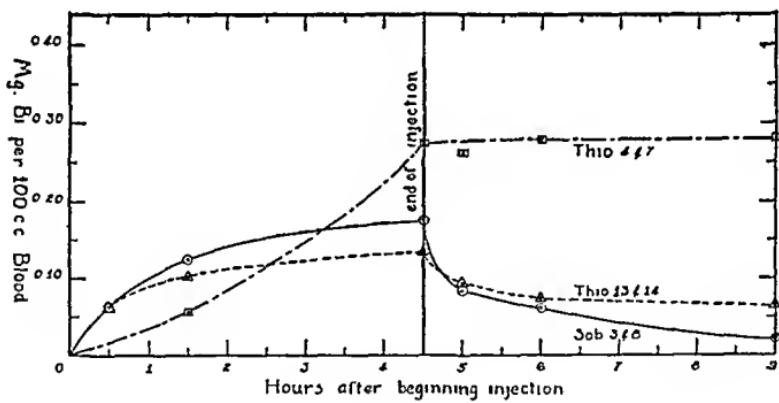
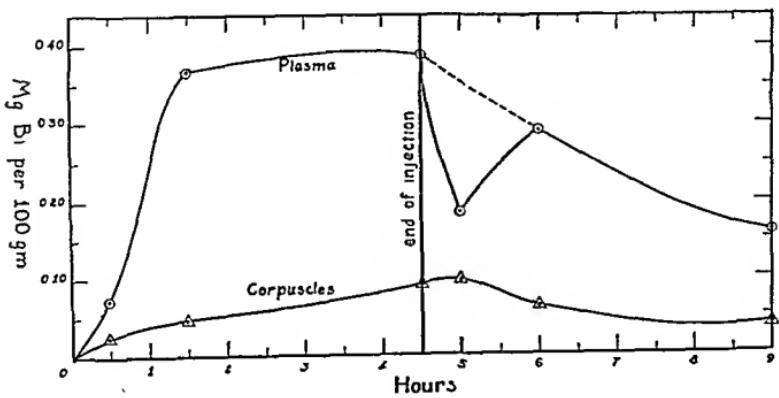


FIG. 3



of hismuth per 100 cc. of whole blood; the medians are plotted as curves in figures 2, 3 and 4.

With the *fast injections*, the fall of hismuth concentration is represented by parabolic curves which are fairly parallel in the four experiments, although the initial concentration was about twice as high for sohisminol injected in 20 minutes as for the thioglycollate injected in 10 minutes. The median concentrations, as shown in figure 2, averaged 0.641 mgm. Bi per 100 cc. at the end of the injection; this had fallen to somewhat less than half (0.295 mgm.) in ten minutes, to about one third (0.220 mgm.) in 30 minutes, to one fourth (0.155 mgm.) in 90 minutes; and to one sixth (0.100) in 4½ hours. By this time the concentration curve was almost horizontal. With the *slow injections* (4½ hours) the median concentration of the six experiments, as shown in figure 2, rose slowly during the injection, in the form of a parabolic curve, to 0.167 mg. Bi per 100 cc. of whole blood, and then descended as a parabolic curve, to three-fifths (100 mgm.) in a half hour, to half (80 mgm.) in 1½ hours, and from there it was almost horizontal. Comparing the median data and curves of the fast and slow injections, it appears that the bismuth concentration at the end of the injection is about four times higher in the fast than in the slow injection, but that the difference is of rather short duration. In about two hours after beginning the injection the blood concentration is about the same for fast and slow injections. The total hismuth equivalent of the blood expressed by the area of the median curves (the product of concentration multiplied by time) is about 19 per cent greater for the fast than for the slow injections, and in this sense and to this degree the fast injections would appear to have greater efficiency (toxic or therapeutic) than the slow injections. However, this difference may not be statistically significant.

Further examination of the six slow injections shows that they fall into three groups, each with two experiments of almost identical concentration curves, shown in figure 3: The two sohisminol animals (3 and 8) and two of the thioglycollate animals (13 and 14) give very similar figures, with the typical parabolic rise of concentration during the injection and the inverse parabolic fall after the injection. The other two thioglycollate experiments (4 and 7), however, gave a materially different curve: The ascending limb of the curve during the injection did not flatten out but reached a median level twice as high as that of the other two thioglycollate animals, and when the injection was stopped the concentration did not fall but continued practically horizontal for the 4½ hours of observation. In other words, the hismuth did not leave the circulation, as it did in the other animals, indicating that the thioglycollate hismuth is liable to pass into nondiffusible, presumably colloidal form in the course of the injections.

Fig. 2 shows the median concentration after fast (solid line) and slow (broken line) injections and their time-relations.

Fig. 3 shows the average bismuth concentrations after slow injection of sohisminol (solid line) and two specimens of thioglycollate (broken and dotted lines).

Fig. 4 shows concentration of the average bismuth blood plasma (solid line) and corpuscles (broken line).

This tendency varies in different specimens, those of injections 4 and 7 coming from one lot, those of 13 and 14 from another.

The concentration curves of plasma and corpuscles were determined separately in two dogs killed by thioglycollate injections (of the same lot as 13 and 14). The median curves (fig. 4) show that thioglycollate bismuth enters and leaves the red blood cells much more slowly than the plasma, so that the bismuth concentration ratio of plasma: corpuscles stands about 4.4:1 at the end of the injection, 4:1 at 4½ hours after the injection, and 1:1 at the end of 24 hours.

*The speed with which the bismuth leaves the blood during the injection* may be calculated by deducting from the quantity injected (4 mgm. per kilogram) that which remains in the blood at the end of the injection (the concentration, in milligram Bi per 100 cc. of blood, multiplied by the normal blood volume, 90 cc. per kilogram of body weight). The percentage of bismuth which has left the blood by the end of the injection is shown in the right-hand column of table 4. It averages 90 per cent for thiobismol injected in 10 minutes; 78 per cent for sobisminol in 20 minutes, 95 per cent for thiobismol in 5 hours, 95.5 per cent for sobisminol in 5 hours.

**THE BISMUTH CONCENTRATION OF RABBIT ORGANS AFTER INTRAVENOUS INJECTION OF BISMUTH.** This is important especially as a basis of comparison with clinical administration, for while it is questionable whether bismuth is therapeutically active after it has been deposited in the organs, their bismuth content reflects the amount of bismuth that has passed through the circulation. Analytical data on clinical material are available for such comparisons, as are also results with intramuscular administration to animals. The analyses of the present series were performed chiefly by G. H. Mangun, with the method used in previous work in this laboratory (11), with the following modification which shortened the time necessary for analysis. Wet ashing was replaced by muffle ashing at 500°C. for 12 to 24 hours, after about 1 to 100 grams of tissue had been evaporated with five times its weight of concentrated nitric acid and a few drops of caprylic alcohol to prevent foaming. The reading was made with a Klett-Summerson photoelectric colorimeter. An average determination can be completed in 24 to 48 hours.

The analyses give the bismuth content as milligrams of bismuth per 100 gram of organ with its natural moisture. Since it may be expected that the dosage would have a very material effect on the concentration, this has also been reduced to a unit dosage, giving a quotient:

$$\frac{\text{concentration} = \text{mgm. Bi per 100 grams of organ}}{\text{dosage} = \text{mgm. Bi administered per kilogram of animal}}$$

For convenience we may designate this as "bismuth retention quotient" (B.R.Q.). The time which elapsed between the injection of bismuth and the death of the animal is another factor which would influence the organ content. From this standpoint, the experiments were grouped under five headings: (A) Acute death, occurring within two hours after injection; (B) Early death, occurring within two hours to one day after injection; (C) Delayed death, occurring > one to

8 days after injection; (D) Late death, occurring > 9 to 24 days after injection; (E) Indefinite survival, killed in 30 to 69 days. A selection of 79 animals for analysis was made from these groups so as to give representation to fast and slow single injections and to repeated injections of various dose ranges of the three bismuth compounds studied. In practically all of these the bismuth was determined in the kidneys, liver, lung and spleen, and in a few animals also in other tissues; the data represent a total of about 350 bismuth determinations. The B.R.Q.'s were calculated and tabulated in relation to the three compounds studied, to their dosage, to the speed of injection, and to single and repeated injections, so as to learn what effect these factors have on the retention of bismuth. Space is not available for the display of the individual data, so that we must be content to summarize the results: When the bismuth-retention quotients are plotted in decreasing sequence against varying doses of the three compounds, these sequences are scattered so irregularly that the two factors, dose and compound, do not appear significantly to alter the quotients. Nor are there consistent and significant differences between fast and slow injections. Repeated injections give somewhat lower figures than single injections, presumably because part of the bismuth is eliminated between the first and last day of injection. The difference, however, is slight, except in one or two instances in which it may have been accidental.

It seems justifiable therefore to base conclusions on the medians of the entire series. These are shown (with the range) in table 5. The concentrations in the kidneys and lungs fall with time in fairly smooth parabolic curves. The liver concentration appears to rise for a day, but this may be accidental since the one-day data are few. It then declines parallel to the kidneys and lungs. According to the data of the smoothed curves, the concentration in kidney, liver and lung falls to about one half between the end of the first and fifth day after injection, to one fourth by the tenth day, to one eighth by the fortieth day. The spleen concentration follows a different course, rising markedly to the fifth day and then declining much more abruptly than that in other organs. It appears therefore that the kidneys and lungs store bismuth rapidly, the liver and spleen more slowly. Table 6 expresses the median concentration of Bi in these organs as percentages of the kidney concentration. The liver ratio is seen to rise progressively with the lapse of time, from 17.1 per cent immediately after injection to 46 per cent after 41 days, so that the liver retains bismuth more tenaciously than the kidneys. The ratios in the lung and spleen rise for 5 days, from 6.8 to 13.8 per cent for the lung, from 11 to 93 per cent for the spleen, but after this time they drop more rapidly than those in the kidney. The tenacious retention therefore appears peculiar to the liver. This may be connected with storage in the reticulo-endothelial system.

The bismuth concentration in the fluids is shown in condensed form in table 7. The number of animals is not sufficient to establish relations for dosage and bismuth compounds, except that these were less than the accidental variations. They are therefore disregarded. The principal deductions are, (1) that the bismuth concentration of the urine and blood sinks to insignificant traces after 6 or

7 days; the urinc concentration averages about 10 times that of the blood, the peritoneal *edema fluid* about one and a half times that of the blood (presumably because of the lower concentration in the blood corpuscles). (2) Till the fourth day the blood concentration averages about one-fifteenth of the liver concentra-

TABLE 5  
*Bismuth concentration in organs of rabbits*

	A. ACUTE DEATH (WITHIN $\frac{1}{2}$ HOUR AFTER INJECTION)	B. EARLY DEATH (2 HOURS TO 1 DAY)	C. DELAYED DEATH (1 TO 8 DAYS)	D. LATE DEATH (9 TO 24 DAYS)	E. SACRIFICED (28 TO 60 DAYS)
Number of ani- mals ana- lyzed.....	12	6	23	13	25
Concentration of Bi (mgm. per 100 grams of fresh organ)					
<i>Kidneys</i>					
Range.....	1.94 - 43.4	4.32 - 8.62	0.57 - 6.8	0.32 - 2.7	0.05 - 1.19
Median.....	8.0	6.12	1.78	0.83	0.27
<i>Liver</i>					
Range.....	0.14 - 8.6	0.52 - 6.93	0.07 - 3.58	0.06 - 0.91	0.03 - 0.28
Median.....	1.37	1.72	0.58	0.296	0.124
<i>Lung</i>					
Range.....	0.05 - 11.9	0.07 - 0.53	0 - 1.09	0 - 0.37	0 - 0.05
Median.....	0.54	0.15	0.245	0.03	0
<i>Spleen</i>					
Range.....	0 - 5.9	trace - 5.38	0 - 6.05	0 - 1.78	0 - 0.235
Median.....	0.89	0.77	1.65	trace	0
Bismuth-retention quotient (concentration divided by dosage as mgm. Bi per kgm. of body weight)					
<i>Kidneys</i>					
Range.....	0.25 - 2.46	0.15 - 1.72	0.077 - 1.36	0.036 - 0.360	0.005 - 0.180
Median.....	0.800	0.671	0.163	0.204	0.076
<i>Liver</i>					
Range.....	0.034 - 0.890	0.082 - 0.176	0.009 - 0.266	0.018 - 0.091	0.003 - 0.087
Median.....	0.084	0.139	0.053	0.054	0.023
<i>Lung</i>					
Range.....	0.016 - 0.313	0.013 - 0.029	0 - 0.232	0 - 0.074	0 - 0.009
Median.....	0.061	0.022	0.020	0.002	0
<i>Spleen</i>					
Range.....	0 - 0.520	trace - 0.359	0 - 1.20	0 - 1.78	0 - 0.067
Median.....	0.101	0.093	0.210	trace	0

tion; the urine concentration averages about a third that of the kidney till the sixth day. After this the concentration falls much more rapidly in the fluids than in the organs. (3) The concentration in the gall bladder *bile* follows a somewhat different course: Only a trace is found at the end of the injections, but it remains at relatively high levels from one to 9 days (the latest that was examined). It averages two-thirds of the liver concentration and in one rabbit the bile

TABLE 6

Median bismuth concentration of rabbits, organs as percentage of kidney concentration

GROUP	MEDIAN TIME OF DEATH	LIVER	LUNG	SPLEEN
A	End of injection	17.1	6.8	11.1
B	1 day	28	2.4	11.0
C	5 days	32.7	13.8	93.0
D	10 days	36	3.6	trace
E	41 days	46	0	0

TABLE 7

Bismuth concentration in fluid of rabbits (at time of death)

TIME OF DEATH	NUM- BER OF ANI- MALS	BISMUTH CONCENTRATION, MM PER 100 CC. (RANGE) AND MEDIAN	BISMUTH CONCENTRATION AS PERCENTAGE OF CONCENTRA- TION IN OTHER FLUIDS OR ORGANS OF SAME ANIMALS (RANGE) AND MEDIAN
Urine in bladder at time of death			
End of injection (8 hours).....	4	(0.38-7.6) = 1.5	(4.6-127) = 27% of kidneys
1 day.....	4	(0.34-3.1) = 1.8	(4-51) = 29% of kidneys
3 to 6 days.....	6	(trace-0.89) = 0.64	(trace-70) = 33% of kidneys
7 to 9 days.....	7	(0-0.67) = 0.05	(0-11.2) = 3% of kidneys
19 days .....	1	trace	trace
28 to 57 days.....	4	0	0
Blood			
End of injection.....	1	0.065	3% of urine; 2.3% of liver
3 and 4 days .....	2	(0.06 and 0.08) = 0.07	(0.6 and 39) = 25% of urine; 7.3 and 9.2 = 8.3% of liver
7, 8 and 10 days.....	3	(trace to 0.195) = trace	trace
26, 56 and 69 days .....	3	(0 to 0.009) = 0	0
Peritoneal edema fluid			
3 to 10 days.....	4	(trace-0.167) = 0.045	4 days = 140% of blood; (13 and 20) = 16.5% of liver
Bile in gall bladder (sobisminal injected)			
End of injection .....	1	trace	
1 day .....	2	(1.9 and 3.03) = 2.5	(43 and 66) = 55% of liver
3, 6 and 9 days .....	3	(0.33 to 3.22) = 1.34	(44 to 230) = 85% of liver

concentration was  $2\frac{1}{3}$  times that of the liver. In a rabbit dying on the first day after injection the bile and urine concentration were about equal: in another dying seven days after injection, the bismuth concentration in bile was ten times that in urine. Sobisminol had been injected in all rabbits whose bile was examined.

*Bismuth determinations in other solid organs.*—A rabbit that died acutely at the end of the slow injection of a large dose of sobisminol (38 mgm. Bi per kilogram) gave the following bismuth concentrations (mgm. Bi per 100 grams): Skeletal muscle, 0.31; bone, 0.333; brain, 0.42; heart muscle, 2.8; intestines, 2.8. The concentration in the heart muscle and small intestines was about nine-tenths that of the liver; in the skeletal muscle and bone, about one tenth. The relatively high Bi content of the heart muscle may have been due to retention of bismuth flocculations in the coronary capillaries. The *intestines* offer a special problem by the precipitation of bismuth sulfide in the mucosa of the large intestines, especially about the spiral valve of the cecum. The small intestines usually contain only a fraction of the liver concentration of bismuth (0.071 mgm. per 100 grams in one day, 0.74 in three days, trace in fifty seven days, one rabbit each). The stomach in a one day rabbit contained 1.71 mgm. per 100 grams. The large intestines, however, contained 13.1 and 13.4 mgm. per 100 grams, in a one day and a three day rabbit, 20 to 200 times as much as the small intestines, 3 to 4 times as much as the kidneys, 4 to 5 times as much as the liver. The mucosa alone is responsible for the high concentration: In a one day rabbit, the concentration in the mucosa of the cecal valve was 17.6 mgm. per 100 grams, that of the underlying intestinal muscle was 1.55 mgm. per 100 grams. Even 57 days after injection the mucosa of the cecal valve of a rabbit gave 7.31 mgm. Bi per 100 grams, 53 times the concentration in the liver. The mucosal deposits are therefore retained with great tenacity, but it may be seen from the color that they undergo chemical changes; up to five weeks they have the brownish black color of bismuth sulfide, and subsequently they bleach slowly to the gray of metallic bismuth. Some of the animals sacrificed after 10 weeks showed no bismuth stain. The *feces* examined in one rabbit, which died three days after injection, had a bismuth concentration, 5.97 mgm. per 100 grams, 30 times the concentration in the urine.

*Comparison with the data of other authors* shows a bismuth retention quotient in rabbits's kidneys and liver identical with ours for the intravenous injections of sobisminol (Hanzlik, Lehman, and Richardson) (5). The *intramuscular injections of watery solutions* (sodium bismuth citrate and tartrate, Leonard *et al.*; sobisminol, Hanzlik *et al.*) give organ concentration of 6 to 14 per cent of equal doses by intravenous injection. With *intramuscular injections of oil suspensions* of the tartrate or citrate (Leonard *et al.*), the difference is not so great, but the organ concentration averages less than half (24 to 45 per cent) of that of equal doses by intravenous injection.

**THE BISMUTH CONCENTRATIONS IN THE ORGANS OF DOGS.** These were studied in nine animals, some with and some without anesthesia, with intravenous injection of 4 to 13 mgm. of Bi per kilogram, in the form of the three compounds,

2 with fast and 7 with slow injections. The data are irregularly distributed among these factors, so that these may be disregarded for this study. The summarized results are shown in table 8. Comparison of this with table 5 in which the results for rabbits are similarly arranged, show that the concentrations in all the organs tend to run several times higher for dogs than for rabbits with equal doses. The cause of this is not known, but it is conceivable that rabbits eliminate bismuth faster, by urine and by precipitation of sulfide in the intestinal mucosa, especially in the earlier periods. This may also explain why the intravenous toxicity of bismuth is somewhat lower with rabbits.

*Total bismuth distribution in the body.*—Table 4 showed that an average of 86

TABLE 8  
Bismuth concentration in organs of dogs

A AT END OF FAST (10 MIN.) INJECTION	B 4½ HOURS AFTER END OF FAST (7 MIN.) IN- JECTION	B		C 2 TO 4 DAYS AFTER SLOW INJECTIONS	E 31 TO 42 DAYS AFTER SLOW INJECTIONS
		4½ HOURS AFTER END OF SLOW (4 HOUR) INJECTION	Number analyzed		
1	1	2	2	3	
Concentration of Bi (mgm. per 100 grams of fresh organ-range and average)					
Kidney	5.40	13.1	8.71 - 26.0 = 17.36	6.00 - 5.30 = 5.65	0.51 - 1.44 = 1.25
Liver	0.92	0.21	0.13 - 0.14 = 0.14	0.58 - 0.71 = 0.65	2.05 - 0.54 = 0.53
Lung		0.19	0.57 - 0.16 = 0.36	0.063	= 0.063 0.026 - 0.054 = 0.050
Spleen	0.25	0.30	0.18 = 0.18	0.31 - 0.38 = 0.35	0.044 - 0.145 = 0.144
Blood	1.06	0.004	0.21 - 0.021 = 0.12	0.014 - 0.013 = 0.014	

Bismuth-retention quotient (concentration divided by dosage, mgm. per kgm. of body weight)

Kidney	1.35	3.28	2.13 - 6.5 = 4.32	0.86 - 0.48 = 0.67	0.10 - 0.25 = 0.11
Liver	0.23	0.053	0.032 - 0.036 = 0.034	0.032 - 0.064 = 0.073	0.010 - 0.107 = 0.041
Lung		0.046	0.143 - 0.037 = 0.090	0.009	= 0.009 0.004 - 0.010 = 0.005
Spleen	0.064	0.075	0.045 = 0.045	0.044 - 0.034 = 0.039	0.009 - 0.039 = 0.011
Blood	0.265	0.023	0.053 - 0.005 = 0.029	0.002 - 0.001 = 0.002	

per cent of the administered bismuth has left the blood stream of dogs in the 10 to 20 minutes occupied by the fast intravenous injection and an average of 95 per cent has left during the slow intravenous injections lasting five hours. The major part of this has gone into the tissues, the remainder being excreted by the urine and feces. Table 9 presents the amounts found in the kidneys, liver, lungs, spleen and blood of dogs after various intervals up to 42 days; and table 10 gives the corresponding and more numerous data for the kidneys, liver and lungs of rabbits. These tables show the actual amounts in each organ, as milligrams of bismuth, and the percentage of the injected dose which this represents. The data for rabbits are shown as medians and range, to save space, without differentiation as to preparation or speed of injection, since it has been found that these do not materially alter the retention.

It will be seen that the percentage distribution of bismuth among kidney, liver and lung is of the same order of magnitude in the dogs and rabbits. During and within two hours after the injection about 3 to 5 per cent of the injected bismuth has gone into the kidneys, 6 to 10 per cent into the liver, 0.4 per cent into the lungs, 0.12 per cent into the spleen. During the remainder of the day the percentage rises somewhat in the kidneys (to 7-10 per cent), and falls somewhat in the liver (to 1-4 per cent) and also in the spleen (to 0.1 per cent). Within the next week the percentage in all the organs has fallen considerably, in the kidney

TABLE 9

*Total bismuth in organs of dogs after intravenous injections (number of animals analyzed, as in table 8)*

	A AT END OF 10 MIN. INJECTION	B 4½ HOURS AFTER END OF FAST INJECTION	B 4½ HOURS AFTER END OF SLOW INJECTION	C 2 TO 4 DAYS AFTER SLOW INJECTIONS	E 34 TO 42 DAYS AFTER SLOW INJECTIONS
Dose of Bi, mgm. per kgm. body weight.....	4	4	4	7 and 11	5, 5 and 13
Total Bi injected, mgm.....	88	70.8	54.4 and 55.6	40.0 and 73.2	24.1, 37.0 and 72.2
Total Bi (mgm.) in organs					
Kidneys (both).....	2.4	6.70	6.56 - 5.14	1.35 - 0.90	0.035-0.405 = 0.182
Liver.....	8.46	1.00	0.378- 0.425	1.09 - 2.06	0.135-1.964 = 0.931
Lung.....		0.268	0.152- 0.80	0.026- —	0.022-0.044 = 0.037
Spleen.....	0.11	0.14	0.047- —	0.028-0.048	0.006-0.027 = 0.018
Blood.....	20.42	1.50	0.257- 2.677	0.09 -0.09	
Percentage of injected Bi in organs					
Kidneys (both).....	2.80	9.46	1.20 - 9.2	3.44 -1.22	0.35 -0.585 = 0.49
Liver.....	9.88	1.41	0.69 - 0.78	2.72 -2.82	0.56 -2.88 = 2.57
Lung.....		0.38	0.33 - 1.4	0.065- —	0.06 -0.10 = 0.09
Spleen.....	0.12	0.19	0.086- —	0.07 -0.065	0.025-0.05 = 0.038
Blood.....	23.1	2.12	0.47 - 4.8	0.22 -0.12	

and liver to about 2.5 per cent, in the lungs to 0.1 per cent, and in the spleen to 0.067 per cent. The decrease continues and in 4 or 5 weeks the kidneys contain about 0.45 per cent, the liver relatively more, (about 1 per cent), the lungs and spleen each about 0.034 per cent. These averages are approximate combinations of the dog and rabbit data.

COMPARISON WITH CLINICAL ORGAN BISMUTH CONCENTRATION.—Analytical data on the autopsy organs of twenty two patients treated clinically by courses of intramuscular injections of bismuth preparations, generally subsalicylate, were published by Sollmann, Cole and Henderson (12). Table 11 shows the median concentration, regardless of dosage, for the principal organs of these patients, in comparison with the concentrations of our rabbits and dogs after single and frac-

tionated intravenous injections. At the end of the single intravenous injection in the animals the organ concentration averaged about twice as high as after a series of courses of intramuscular injections in the patients. The kidney concentration fell to about half the clinical level in a week; but the concentration in the other organs remained about the clinical level for two months. The intravenous dosage was higher than in a single clinical injection, but the median of the total clinical dosage is about 15 mgm. Bi per kilogram of body weight, which is two or three times the median intravenous dosage.

The patients averaged a kidney content of 4.5 mgm. per 100 gram for each gram bismuth injected, equivalent in a 70 kgm. patient to a B.R.Q. of 4.5:15 =

TABLE 10  
*Total bismuth in organs of rabbits after intravenous injections*

	GROUP A	GROUP B	GROUP C	GROUP D	GROUP E
	Time of death after injection				
	Within 2 hours	2 hours to 1 day	2 to 8 days	0 to 24 days	25 to 60 days
Number of animals included.....	8	3	10	12	23
Median dose of Bi (mgm. per kgm.).	11.5	6.0	7.0	5.5	5
Total Bi (mgm.) in organs, average and range					
Kidneys (both).....	2.04 (0.34-6.08)	1.299 (0.54-1.88)	0.513 (0.13-1.83)	0.220 (0.043-0.57)	0.072 (0.013-0.131)
Liver.....	2.41 (0.163-6.83)	0.653 (0.52-0.78)	0.452 (0.055-1.07)	0.231 (0.034-0.050)	0.116 (0.020-0.298)
Lungs.....	0.181 (0.015-0.71)	0.033 (0.019-0.040)	0.019 (0.041)	0.014 (0.078)	0.0005 (0-0.006)
Percentage of injected Bi in organs (average)					
Kidneys (both)....	4.95	7.08	2.60	1.208	0.448
Liver .. ...	5.75	3.72	2.28	1.535	0.838
Lungs .....	0.41	0.183	0.095	0.079	0.034

0.3. This quotient is less than that at the end of intravenous injection in rabbits (median 0.8), and even after one day (median 0.65). It is about equal to that of five days after intravenous injection (median 0.35). Beyond these times it rises above that of the intravenous quotient, which has the medians of 0.18 in 10 days, 0.1 in 20 days, 0.08 in 40 days. The intravenous injection therefore secures a higher initial concentration, but beyond five days after a single intravenous injection the concentration falls below that produced by the intramuscular courses.

From a smoothed curve of the data of table 5, one may calculate the amount of bismuth that must be given by a single intravenous injection to secure a concentration of 3.33 mgm. Bi per 100 gram of kidney, at any time after the injection: Immediately after injection, this approximates 4 mgm. per kilogram; for one day after injection, 5 mgm. per kilogram would need to be injected; to

have this concentration on the fifth day would require the initial injection of 10 mgm. per kilogram; for the tenth day, 20 mgm.; for the twentieth day, 33 mgm.; for the fortieth day, 40 mgm. Even the smallest of these quantities, 4 mgm. per kilogram of 280 mgm. for a 70 kgm. man would appear far too dangerous. Klauder (3) injected patients twice a week intravenously with colloidal bismuth hydroxide, equivalent to 1 mgm. of bismuth per kilogram of body weight, without untoward effects, but the acute fatality of Curtis occurred with about 0.25 mgm. of bismuth (as sodium bismuth tartrate) per kilogram of body weight.

**ANTISYPHILITIC EFFICIENCY OF INTRAVENOUS BISMUTH PREPARATIONS.**—The question whether the extra high bismuth concentrations that are secured temporarily by intravenous injection add materially to the efficiency of bismuth

TABLE 11

*Median concentration of bismuth (mgm. per 100 grams of fresh tissue), regardless of dosage*

SERIES	KIDNEY	LIVER	LUNG	SPLEEN	BLOOD
Patients after clinical courses of intramuscular injections.....	3.33	0.68	0.085	0.155	0.05
Intravenous, rabbits					
End of injection.....	7.67	1.37	0.538	0.893	0.065
1 day to 1 week.....	1.69	0.520	0.250	1.40	0.07
9 to 24 days.....	0.831	0.164	0.038	trace	trace
28 to 60 days.....	0.268	0.182	0	0	0
Intravenous, dogs					
End of injection.....	15.4	0.92		0.25	1.06
4 hours after injection.	13.1	0.140	0.185	0.300	0.094
2 and 4 days.....	5.7	0.642	0.063	0.347	0.014
34 to 42 days.....	1.250	0.533	0.050	0.144	

treatment of syphilitic infections can only be answered by direct experiments. Dr. Hanzlik has started to investigate this on rabbits.

#### SUMMARY AND CONCLUSIONS

Three special water-soluble bismuth compounds—sodium bismuth citrate, sodium bismuth thioglycollate, and sobisminol, were injected intravenously into rabbits and dogs, comparing the “drip method” of very slow injection of dilute solutions extending continuously over four to ten hours, with injection of the same doses at the usual rate of ten to thirty minutes; the entire amount being introduced either on one day, or spread over several successive days, up to five. The toxicity of the citrate and of sobisminol with the very slow injections was about half that with the ordinary speed; this does not apply to the thioglycollate solution (presumably because this is more unstable).

Spreading the total dosage over several successive days decreases somewhat

the nephritic toxicity but increases the liability to acutely fatal reactions in rabbits, probably by thrombosis from traumatic vascular inflammation.

Acute fatalities during or within a few minutes after the injection occur also with single injections, about twice as frequently with fast injections (about 20 per cent of all fatalities) than with slow (about 10 per cent); but they are 2 to 4 times more frequent with repeated injections. They have little or no relation to the dosage and present the phenomena of colloidoclastic shock, while the ordinary bismuth phenomena are nephritis.

Death from bismuth nephritis occurs in several hours to 24 days after the injection, the time being generally inverse to the dose; for instance, half of the dosage that kills in one day is fatal after 9 days for slow, after 5 days for fast injections.

The fatal dose by intramuscular injection appears to be of the order of 5 to 10 times higher than that by slow intravenous injection, for rabbits.

The fatal dose of bismuth by intravenous injection averages somewhat smaller for dogs than for rabbits. The symptoms in dogs are uremic, without the bloody diarrhea, peripheral neuritis or encephalopathy that are conspicuous in poisoning by alkyl bismuth.

The sojourn of the injected bismuth and its concentration in the blood were studied on dogs. With the fast injections the concentration falls rapidly as a parabolic curve. With the slow injections it rises parabolically during the injection and falls similarly when the injection ceases. With fast injection the maximal concentration in the blood is about four times as high as that attained by slow injection, but by the end of two hours the concentration is about the same in both cases. The product of average concentration and total time is about one-fifth higher with fast than with slow injection of a given dosage.

The bismuth concentration of the organs varied directly with the dose and inversely with the time elapsed since the injection. The nature of the bismuth compound and the speed of injection are not significant. The concentrations in the kidneys (which are richest in bismuth) and in the lungs fall with time as fairly smooth parabolic curves. The concentration in the liver falls more slowly, so that its average ratio to that in the kidneys rises from 17 per cent on the first day, to 46 per cent at 41 days. The concentrations in the blood and urine sink to insignificant traces after 6 or 7 days; in the bile the Bi rise is slower and more persistent.

The bismuth concentration in the organs of dogs averages several times higher than for rabbits, with the same dosage and time. This suggests slower elimination of Bi by dogs (by the urine and by precipitation of sulfide in the intestinal mucosa) and this would explain the higher toxicity for dogs.

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# THE FATE OF CERTAIN SYMPATHOMIMETIC AMINES IN THE BODY

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The fate of amphetamine in the body—that it is partially excreted as such (1, 2, 3), partially inactivated in the liver (4, 3) and deaminated by ascorbic acid (5) but not by amine oxidase (6, 7, 4)—seems well established. The purpose of the present investigation has been to determine whether a number of compounds related to amphetamine are excreted in the urine or totally inactivated in the body, and to examine these findings in the light of previous studies.

**PROCEDURE.** The amines studied in this work were

1.  $\beta$  phenyl-*n*-propylamine
2.  $\gamma$  phenyl-*n*-propylamine
3.  $\alpha$  methyl- $\gamma$ -phenylpropylamine
4.  $\alpha$  phenyl-*n*-propylamine
5.  $\beta$  methyl- $\beta$ -phenylisopropylamine
6.  $\alpha$  isopropyl- $\beta$ -phenylethylamine
7.  $\alpha$  methyl- $\beta$ -phenylisopropylamine

The method used to estimate the presence of these amines in urine was that first described by us (4) with some minor modifications (5). This has proved satisfactory for the present investigation. The only deviation from the method as previously described has been in the final determination. In this study we have used duplicate controls of 0.1 and 0.01 mgm. amine coupled with *p*-nitrobenzene-diazonium chloride and extracted with butanol. The galvanometric deflections for these controls have been plotted as the logarithmic function of the concentration on 2 cycle semi-log paper, a straight line drawn through the points, and the concentration of the sample read as the arithmetic coordinate where the galvanometric value intersected the curve. This has the advantages of doing away with a constant reference curve, as has been used in the past, and the corrections to such a curve when control determinations vary from it only slightly.

**EXPERIMENTAL.** *Amines excreted by the kidneys.* Ten milligram doses of compounds 3, 4, 5, 6, and 7 were given by mouth to 31 subjects who collected their total urine output for the first and second 24 hour periods following ingestion of the drug. The subjects were given the compounds in 20 cc. of water and directed to rinse the contents into a glass of water, drink that and follow it with half a glass of water. This was taken about half an hour before breakfast. Since most of the experiments were done during summer months, the urine volumes were often low. In every instance the compounds were excreted in considerable amounts, varying somewhat with the subject and the amine given.

Table 1 gives the results of excretion of 10 mgm. doses of  $\alpha$  methyl- $\gamma$ -phenyl-

propylamine (sulfate) by 10 subjects. Table 2 represents data on the excretion of 10 mgm. doses of  $\alpha$  phenyl-*n*-propylamine,  $\beta$  methyl- $\beta$ -phenylisopropylamine,  $\alpha$  isopropyl- $\beta$ -phenylethylamine and  $\sigma$  methyl- $\beta$ -phenylisopropylamine. Common to all of these is the fact that excretion of the amine is not related to the total urine volume within normal limits. The duration of excretion of determinable amounts of the amines was found to be about 48 hours, with one exception. Other trials have shown them to be present in the urine within 3 hours or less after the compound was swallowed.

However, there is some difference in the rate and extent to which these amines are excreted. From 20 to 54 (average 40.4) per cent of a given dose of  $\alpha$  methyl- $\gamma$ -phenylpropylamine was excreted in 24 hours. Figuring from averages, this represents about  $\frac{2}{3}$  of the total amount (5.9 mgm.) excreted in 48 hours. In the case of  $\alpha$  phenyl-*n*-propylamine 63 to 95 (average 78.9) per cent was excreted

TABLE I

*The excretion of 10 mgm. of  $\alpha$  methyl- $\gamma$ -phenylpropylamine taken orally by man*

SUBJECT	1ST 24 HOUR PERIOD		2ND 24 HOUR PERIOD		TOTAL 48 HOUR PERIOD	
	Urine volume	Per cent excreted	Urine volume	Per cent excreted	Urine volume	Per cent excreted
R. D.....	1024	37.0	1500	26.4	2524	63.4
W. S.....	850	32.2	1250	22.0	2100	54.2
L. K.....	560	21.2	920	20.2	1480	41.4
O. E.....	625	36.6	655	11.8	1280	48.4
J. C.....	600	52.8	560	6.8	1160	59.6
W. R.....	860	47.5	975	30.1	1835	75.6
W. H.....	575	35.0	640	10.2	1215	45.2
B. B.....	1100	44.0	770	33.2	1870	77.2
A. P.....	980	44.1	550	22.4	1530	66.5
R. E.....	1098	54.2				
Average.....		40.4		20.4		59.1

within 24 hours. This comprises almost the total amount excreted; only an average of 2.8 of the total 81.7 per cent was excreted in the second 24 hour period.

The range of deviation from the mean was greatest for  $\beta$  methyl- $\beta$ -phenylisopropylamine, being from 10 to 39.5 per cent excreted within the first 24 hours. The average of 24 per cent is  $\frac{2}{3}$  of the total amount, 30 per cent, excreted during the experiment. The excretion of  $\alpha$  isopropyl- $\beta$ -phenylethylamine was the least of any of the compounds, being only about 20 per cent within 48 hours. In this instance, however, the duration of excretion of determinable amounts of the amine was 72 to 76 hours following ingestion. The total amount of  $\sigma$  methyl- $\beta$ -phenylisopropylamine excreted over 48 hours (88.2 per cent average) was but slightly greater than for  $\alpha$ -phenyl-*n*-propylamine (81.7 per cent average) yet the rate of excretion of the former was much slower than the latter. In the case of  $\sigma$  methyl- $\beta$ -phenylisopropylamine about  $\frac{2}{3}$  of the total amount (60.3 per cent) was excreted within 24 hours, whereas almost all (97 per cent) of the  $\alpha$  phenyl-*n*-

propylamine was excreted during the first 24 hours. Acid hydrolysis of the urine did not increase the recovery of any of the nmines.

TABLE 2

*The excretion of certain sympathomimetic amines by man. 10 mgm. of each was administered in solution orally*

SUBJECT	1ST 24 HOUR PERIOD		2ND 24 HOUR PERIOD		TOTAL 48 HOURS	
	Urine volume	Per cent excreted	Urine volume	Per cent excreted	Urine volume	Per cent excreted
$\alpha$ phenyl-n-propylamine						
R. K. ....	530	95.0	550	3.6	1080	98.5
S. B. ....	1360	88.0	925	1.8	2285	89.8
I. E. ....	415	73.7	690	3.6	1105	77.3
B. S. ....	1210	63.4	1900	1.3	3110	64.7
H. G. ....	590	74.7	740	1.6	1330	76.3
Average.....	78.9			2.8		81.7
$\beta$ methyl- $\beta$ -phenylisopropylamine						
R. S. ....	500	10.0	655	2.0	1155	12.0
E. K. ....	1030	33.0	1720	13.2	2750	46.2
K. N. ....	640	12.4	850	9.8	1490	22.2
C. L. ....	375	10.0	610	13.4	985	23.4
M. O. ....	750	39.5	520	12.5	1270	52.0
T. L. ....	710	39.4	760	8.1	1470	47.5
Average.....	24.0			6.0		30.0
$\alpha$ isopropyl- $\beta$ -phenylethylamine						
M. A. ....	1640	11.0	1640	5.5	3280	16.5
D. B. ....	700	9.3	550	12.8	1250	22.1
L. B. ....	830	14.1	900	10.8	1730	24.9
G. M. ....	400	9.6	600	6.4	1000	16.0
M. S. ....	765	10.3	578	9.2	1343	19.5
Average.....	10.9			8.9		19.8
$\alpha$ -methyl- $\beta$ -phenylisopropylamine						
F. B. ....	1000	69.5	1190	36.1	2190	95.6
A. E. ....	420	56.0	340	24.6	760	80.5
F. B. ....	610	77.3				
R. L. ....	850	37.4	760	27.9	1610	65.3
B. T. ....	1335	71.6	1205	23.3	2541	94.9
Average.....	60.3			27.9		88.2

Using the Warburg respirometer and the technic described previously (8) we found that none of these five compounds was oxidatively deaminated in the presence of amine oxidase. This has also been found to be the case for amphetamine (6, 7, 4).

*Amines not excreted by the kidneys.*<sup>1</sup> Fifteen milligram doses of  $\beta$ -phenyl-*n*-propylamine and  $\gamma$  phenyl-*n*-propylamine were given orally to 8 subjects. Ten to 20 mgm. doses of each were injected subcutaneously daily for several days into 14 dogs. Neither of the compounds was excreted in the urine in more than an occasional trace by either the human subjects or the dogs. Thus the factor of destruction of the compounds before their absorption into the body was ruled out.

Since these 2 amines were not normally excreted following their administration, it was thought desirable to see if the dogs could be made to excrete these chemicals. To accomplish this 3 experiments were tried: 1) to inactivate the amine oxidase in the body, if possible, 2) by means of a substance (CCl<sub>4</sub>) toxic to the liver and possibly other organs as well to impair their function, 3) to impair liver function alone.

Bernheim reported (9) that phenylhydrazine when added in concentration of  $4.5 \times 10^{-4}$  M to an amine oxidase preparation *in vitro* would totally inhibit the oxidative deamination of isoamylamine as substrate for the enzyme. In a study of the enzymic inactivation of substituted phenylpropylamines (8) one of us found that these two compounds were deaminated by amine oxidase. An attempt was made, then, to inject phenylhydrazine into dogs to inhibit if possible the amine oxidase and so cause  $\beta$  phenylpropylamine to be excreted.

In this experiment 4 dogs were injected subcutaneously daily with 10 mgm.  $\beta$  phenyl-*n*-propylamine and their 24 hour urine specimens collected and analyzed for the amine. Three days of control determinations were made to determine whether or not the compound was excreted. Total red and white blood cell counts, differential cell counts and hematocrit were determined on the controls and following the phenylhydrazine administration. Following the control periods the dogs were weighed and 100 mgm. of phenylhydrazine hydrochloride per kilo of body weight was injected subcutaneously in a volume of distilled water sufficient to dissolve it. The dogs usually vomited within an hour and appeared sick. The injections of the amine, collection of urine and blood counts were continued for from 4 to 6 days following the phenylhydrazine injections. During this time no  $\beta$  phenylpropylamine appeared in the urine though the dogs manifested severe phenylhydrazine poisoning. One of them, T, died 1 week following the injection of phenylhydrazine. Table 3 summarizes some of the findings in this experiment. In every instance, following the phenylhydrazine there resulted a severe hemolytic anemia, a rapid rise in the leukocyte count and a fall in the hematocrit readings. The differential cell counts showed many immature cells of both red and white series. Representative of the differentials is one day's count on dog C: Total w.b.c. 34,900, segmented neutrophils 20, nonsegmented neutrophils 58, meta myelocytes 6, myelocytes 4, promyelocytes 2, blasts 1, eosinophils 2, lymphocytes 7, erythroblasts 9 and proerythroblasts 3/100 w.b.c. There were numerous showers of platelets, polychromatophilia and anisocytosis. Urine from all the dogs was port red in color due to the destruction of erythrocytes and that from dogs S and T tended to form a loose

<sup>1</sup> Normally under the conditions of this experiment.

clot on standing in the refrigerator several days. It is doubtful whether we could have exceeded this dose of phenylhydrazine and have had any of the dogs live. It seems certain, then, that not enough of the drug could be given to animals to inactivate or even inhibit the amine oxidase to the point that  $\beta$ -phenyl-n-propylamine was excreted by the animals.

Carbon tetrachloride is known to produce liver damage in dogs although its effect on the kidney is probably not so marked unless excessive amounts are given (10). An experiment similar to that for phenylhydrazine was performed, substituting in its stead  $CCl_4$  and omitting the cell counts. Four dogs were used in this experiment and the condensed data are given in table 4. From the table it may be seen that in every instance  $CCl_4$  in doses of 30 to 50 cc. orally caused  $\beta$ -phenyl-n-propylamine to be excreted; this amounted to as much as 35 per cent of a given dose in one instance, dog D.

TABLE 3

*The result of an attempt to produce excretion of  $\beta$ -phenyl-n-propylamine, 10 mgm. subcutaneously daily, by the subcutaneous administration of a single injection of phenylhydrazine hydrochloride*

DOG	AMOUNT OF PHENYLHY- DRAZINE	ERYTHROCYTES		LEUKOCYTES		HEMATOCRIT		EXCRETION OF AMINE	
		Before	After	Before	After	Before	After	Before	After
	mgm. per kgm.	millions	millions	thousands	thousands	per cent	per cent		
C	75	7.40	1.00	9.15	37.20	50.6	10.3	0.00	0.00
E	100	7.33	1.76	0.50	26.65	49.5	27.5	0.00	trace
S	100	6.17	2.73	12.40	35.25	44.0	30.0	0.00	0.00
T*	100	4.48	10.6			44.0		0.00	trace

\* Died 1 week after phenylhydrazine.

Before and after pertain to the injection of phenylhydrazine. The data given for after the injections are maximal determined values.

This effect of  $CCl_4$  is probably a non-specific one, inhibition to the amine oxidase system occurring along with a general impairment of liver function. It was shown by Beyer and Skinner (4) that carbon tetrachloride administered to dogs caused them to excrete all of a given dose of benzedrine (amphetamine), and it has been amply confirmed that this compound is not inactivated by amine oxidase.

Wells (11) has shown that hydrazine, in doses of 50 mgm. per kilo subcutaneously, produces in dogs an almost specific central zone parenchymatous degeneration of the liver lobules without affecting other organs of the body. If  $CCl_4$  could non-specifically inhibit amine oxidase along with the production of a parenchymatous degeneration of the liver and possibly other organs, it seemed possible that similarly hydrazine could be used to produce only a liver damage, thus in a measure evaluating the importance of the liver in the inactivation of these amines.

Three dogs each in two series were placed on daily subcutaneous injections

TABLE 4

*The effect of carbon tetrachloride on the excretion of  $\beta$  phenyl-n-propylamine, 10 mgm. subcutaneously daily, by dogs*

24 HOUR PERIOD	URINE VOLUME	MOM. EXCRETED	24 HOUR PERIOD	URINE VOLUME	MOM. EXCRETED
Dog D			Dog M		
2nd	625	0.00	3rd	300	0.00
3rd	590	0.00	4th	290	0.00
4th	30 cc. $CCl_4$ orally		5th	50 cc. $CCl_4$ orally	
6th	420	1.77	7th	145	0.29
8th	640	0.94	8th	355	1.63
12th	875	3.50	9th	195	trace
13th	570	2.52	10th	185	trace
Dog N			Dog O		
3rd	335	0.00	1st	755	0.00
4th	560	0.00	2nd	480	0.00
5th	50 cc. $CCl_4$ orally		3rd	50 cc. $CCl_4$ orally	
7th	415	0.42	4th	730	2.14
8th	440	0.41	5th	50 cc. $CCl_4$ orally	
9th	655	trace	6th	385	0.65
10th	380	trace	7th	855	trace

TABLE 5

*The effect of hydrazine on the excretion of  $\beta$  phenyl-n-propylamine and  $\gamma$  phenyl-n-propylamine by dogs*

24 HOUR PERIOD	URINE VOLUME	MOM. AMINE EXCRETED	URINE VOLUME	MOM. AMINE EXCRETED	URINE VOLUME	MOM. AMINE EXCRETED
$\beta$ phenyl-n-propylamine, 20 mgm. injected subcutaneously, daily						
	Dog P		Dog Q		Dog R	
2nd	390	0.00	215	0.00	765	trace
3rd	335	0.00	260	0.00	365	trace
4th	40 mgm. hydrazine sulfate per kgm. subcutaneously in 2 injections					
5th	655	0.32	355	0.65	1235	2.50
6th	150	0.57	335	0.92	225	2.35
7th	170	0.78	280	0.82	625	1.41
8th	125	0.25	380	1.14	650	0.94
$\gamma$ phenyl-n-propylamine, 10 mgm. injected subcutaneously, daily						
	Dog U		Dog W		Dog X	
2nd	225	0.00	300	0.00	315	0.00
3rd	100	0.00	250	0.00	320	0.00
4th	40 mgm. hydrazine sulfate per kgm. subcutaneously in 2 injections					
6th	235	0.24	1050	0.42	265	0.26
7th	275	0.28	235	1.95	455	0.23

of 20 mgm.  $\beta$  phenyl-*n*-propylamine in one series, 10 mgm. of  $\gamma$  phenyl-*n*-propylamine in the other. Total 24 hour urine specimens were collected and tested for the respective amines. Neither series of dogs excreted the amines normally with the exception of dog R which excreted about 2  $\mu$ /cc. of  $\beta$  phenyl-*n*-propylamine. After the control periods of 3 days the dogs were injected subcutaneously with 40 mgm. hydrazine sulfate per kilo in aqueous solution given in two divided doses at 6 hour intervals. It may be seen from table 5 that this dose of hydrazine caused the excretion of both  $\beta$  phenyl-*n*-propylamine and  $\gamma$  phenyl-*n*-propylamine by each dog of the respective series. It is not surprising that the excretion of the amines following hydrazine is no greater than that found. Bhagvat, Blaschko and Richter (12) have reported amine oxidase to be widely distributed in most of the organs of the body. If the distribution were equal throughout the organism, one would hardly expect an inhibition in or even elimination of that enzyme from one organ so to decrease the detoxication of these amines that they appear as such in the urine. Actually, it would seem that the liver is a principal site of inactivation of these amines whether or not it be entirely by amine oxidase.

**INTERPRETATION.** These results together with what has been found to be true for other similar sympathomimetic amines may be taken to establish certain fundamental concepts of the way an organism rids itself of these agents. The compounds  $\alpha$  methyl- $\gamma$ -phenylpropylamine,  $\alpha$  phenyl-*n*-propylamine,  $\beta$  methyl- $\beta$ -phenylisopropylamine,  $\alpha$  isopropyl- $\beta$ -phenylethylamine and  $\alpha$  methyl- $\beta$ -phenylisopropylamine together with amphetamine have structural and physiological properties in common. None of these compounds have a hydroxyl group on the ring, none of them have an amino group on the terminal carbon atom of the side chain, otherwise they are quite dissimilar except that they are primary amines.

For the present at least one factor in therapeutics, mode of administration, can be correlated very well with the mode of elimination of these compounds. In this particular group of amines the position of the amino group on the side chain determines whether the compound shall be active orally and excreted by the kidneys, or inactive orally and totally inactivated in the body enzymically or otherwise. The compounds that do not have the amino group on the terminal carbon atom are active orally, not because they are not broken down by the digestive juices, but because on being taken into the body they are not deaminated at once when brought by the portal system to the liver. This being the case they are then carried to all parts of the body. Since they remain in the blood stream, at least to some extent, for a long period of time they are cleared from the blood by the kidneys and appear in the urine. Part of the drug remaining in the tissues is inactivated by some system as the ascorbic-dehydroascorbic acid system (5, 13). This, probably together with differences in rate of diffusion and affinity for the tissues, accounts for the differences in rate and extent of excretion of these amines.

Confirming this interpretation, these compounds having an amino group on the terminal carbon atom have no physiological effect when taken orally. Also, they do not appear in the urine as such even when injected subcutaneously. It seems likely then that, administered orally, instead of being broken down in the digestive tract the compound is absorbed, deaminated to some extent in the intestinal wall, where amine oxidase has been shown to be present, and the rest brought to the liver where the enzymic oxidation is completed. Bearing out this point are the experiments showing that even when these compounds,  $\beta$  phenyl-*n*-propylamine and  $\gamma$  phenyl-*n*-propylamine, are injected subcutaneously they are excreted to some extent when the function of the liver is impaired by carbon tetrachloride or hydrazine. These observations point to the liver as being a chief organ for the inactivation of these amines. Amine oxidase and the ascorbic acid system are relatively slow acting when saturated with a proper substrate *in vitro*. It might be that massive doses of one of these amines given orally would partially escape deamination and appear in the urine in very small amounts.

#### SUMMARY

Sympathomimetic amines having no hydroxyl group on the benzene ring are excreted if the primary or secondary amino group is not on the terminal carbon atom of the side chain, for only in that position is it deaminated by amine oxidase. If the liver function is impaired by  $CCl_4$  or hydrazine, those compounds having a primary or secondary amino group on a terminal carbon atom are also excreted to some extent.

An hypothesis has been presented that the oral efficacy and the excretion of these amines is dependent on whether the compound brought to the liver has its amino group in a position on the side chain where it can be deaminated by the amine oxidase and possibly other systems, normally.

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# THE ACTION OF CERTAIN SULFONAMIDES, SULFONES AND RELATED PHOSPHORUS COMPOUNDS IN EXPERIMENTAL TUBERCULOSIS

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Rich and Follis (1) first called attention to the inhibitory action of sulfanilamide for the tuberculous process in guinea pigs. They noted retardation of the progress of infection only if treatment was begun simultaneously with the infection and the drug administered in doses of 1250 mgm. per kilogram per day. Such dosage is at the toxic level, as shown by the death of about 60 per cent of their animals from the toxic effects of the drug within the experimental period of five to six weeks. Later they extended their observations to experimental bovine infection in rabbits and showed that 750 mgm. of the drug given daily per kilogram reduced the extent of tuberculous involvement in the lungs and kidneys, but a dosage of 500 to 600 mgm. per kilogram had no appreciable effect. Significantly 750 mgm. of sulfapyridine per kilogram similarly given had no effect. Examination of the blood at various intervals showed considerably higher blood levels with sulfanilamide than with sulfapyridine (2). Several reports have appeared since then, some confirming the foregoing findings in part or in whole (3-6) and others failing to do so (7-9). Greey, Boddington, and Little (5) obtained a favorable effect in guinea pigs with sulfanilamide but not with prontosil, the dimethyl derivative of disulfanilamide, or with diaeetyl-diaminodiphenylsulfone. The doses used were within the toxic range, for half of their treated animals died within four weeks of treatment. Corper *et al.* (9) suggest that the apparently favorable effect obtained with large doses of sulfanilamide is due to the toxic effect of the drug and not to the retardation of the tuberculous process.

Further trials with sulfapyridine gave variable results. Feldman and Hinshaw (10) and Birkhaug (11) obtained retardation of the tuberculous process in guinea pigs, while Heise and Steenken (12), Steinbach and Duca (13), Flippin and associates (14), and Musebenbeim and coworkers (15) failed to obtain favorable effects. Karlson and Feldman (16) treated rabbits inoculated with avian tubercle bacilli with sulfapyridine with negative results.

An inhibiting action by sulfanilamide on the growth of the tubercle bacillus *in vitro* was reported by Ballon in a concentration of 100 mgm. per cent (17, 18), while Follis could obtain no such effects while using the synthetic Proskauer and Beek medium. Follis was able to demonstrate an inhibiting effect for sulfapyridine in a concentration of 50 mgm. per cent (19).

A much stronger inhibiting action *in vivo* was obtained by Rist, Bloch, and

Hamon (20) for 4-4'-diaminodiphenylsulfone than for sulfanilamide on intravenous avian bacillus infection in rabbits. More recently, while this work was in progress, two very favorable reports have appeared by Feldman, Hinshaw, and Moscs (21) on the treatment of experimental tuberculous infection in guinea pigs with the sodium salt of *P-P'*-diaminodiphenylsulfone-*N-N'*-dextrose sulfonate (Promin). In the first report the treated animals survived longer than the controls, and the extent of tuberculous involvement, judged by the amount of tuberculosis in the spleens, was less in the treated animals than in the controls. It was stated, however, that a complicating nutritional disorder developed in the course of the experiment, seemingly an ascorbic acid deficiency, and this may have influenced the results. In a second communication (22) these authors reported equally good results even when treatment with promin was delayed until six weeks after infection. Here again the spleen alone was used to evaluate the extent of tuberculous involvement. It would seem that these workers were dealing with a low grade mild infection, apparently limited for the most part to the spleen and glands. The drug was fed with the diet at a 1 per cent level. Blood examination, they reported, showed an average of 4.3 mgm. per cent of the drug, with a minimum of 2.5 and a maximum of 8.6 mgm. per cent. No toxic effects from the drug were noted in the first report, but sulfhemoglobinemia, reticulocytosis, and possibly anemia are said to have occurred in the second series of experiments. Since promin is a derivative of diaminodiphenylsulfone, a drug which was under study in the work reported here, it seemed desirable to include it in this investigation.

The present experiments were undertaken in the belief that the work cited above indicates sufficiently definitely that the sulfonamides offer enough of a lead to warrant more extensive investigation of the problem. Accordingly, a systematic study was made of the effects of a series of sulfonamides, sulfones, and related phosphorus compounds on the growth of the tubercle bacillus *in vitro*, and the compounds appearing to have good tuberculostatic action *in vitro* were submitted for therapeutic tests in experimental animals. It should be stated at once that neither is the presence of tuberculostatic action of a drug *in vitro* proof for, nor is its absence proof against therapeutic efficiency in experimental animals. Tuberculostatic action, however, may be used as a guide in selecting from a large number of drugs those which are likely to be most promising.

**TUBERCULOSTATIC ACTION IN VITRO.** The tests for the inhibiting action of drugs on the human tubercle bacillus were made with graded concentrations of the drug in 50 cc. sterile glycerine broth inoculated on the surface with a loopful of a two to three weeks' culture of *H 37*. In several instances the human strain *A 27* was also used with no noticeable difference in results. Pyrex glass Erlenmeyer flasks of 125 cc. capacity were used throughout. The flasks were incubated at 37°C. for two weeks or longer, until the whole surface in the controls was covered with growth. The minimum concentration of the drug which gave only slight or no growth was taken as the effective inhibiting concentration.

The results of this study are shown in table 1. Of the sulfonamides good inhibition was obtained at a level of 20 mgm. per cent with sulfanilyl sulfanilamide

and sulfapyridine, both of which were more active than sulfanilamide. Weight for weight promin was about equally effective. Inhibition at a level of 10 mgm.

TABLE I  
Tuberculostatic action

NUMBER	COMPOUND*	CHEMICAL FORMULA	MOM. PER CENT	
			Good growth	Slight or none
1	Sulfanilic acid	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> SO <sub>3</sub> OH	500	
2	Sulfanilamide	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> SO <sub>3</sub> NH <sub>2</sub>	20	50
3	<i>N'</i> -(4 aminophenyl) sulfanilamide (23)	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> SO <sub>3</sub> NH C <sub>6</sub> H <sub>4</sub> NH <sub>2</sub>	20	
4	<i>N</i> <sup>4</sup> -Sulfanilyl sulfanilamide (23)	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> SO <sub>3</sub> NH C <sub>6</sub> H <sub>4</sub> SO <sub>3</sub> NH <sub>2</sub>	10	20
5	Sulfanilylaminooctanol (23)	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> SO <sub>3</sub> NH C <sub>6</sub> H <sub>4</sub> OH	100	
6	Sulfanilylaminobromethane <sup>1</sup>	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> SO <sub>3</sub> NH C <sub>6</sub> H <sub>4</sub> Br	20	
7	4-Hydroxylaminobenzene sulfonamide <sup>2</sup>	NHOH C <sub>6</sub> H <sub>4</sub> SO <sub>3</sub> NH <sub>2</sub>	20	
8	4-Aminobenzoic acid	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> COOH	20	
9	4-Hydroxylaminobenzoic acid <sup>3</sup>	NHOH C <sub>6</sub> H <sub>4</sub> COOH	20	
10	Sulfapyridine	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> SO <sub>3</sub> NH C <sub>6</sub> H <sub>3</sub> N	10	20
11	Sulfathiazole	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> SO <sub>3</sub> NH C <sub>6</sub> H <sub>2</sub> NS	1	5
12	Sodium Sulfadiazine <sup>4</sup>	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> SO <sub>3</sub> NH C <sub>6</sub> H <sub>2</sub> N <sub>3</sub>	4	10
13	4-4'-Diaminodiphenylsulfide	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> SC <sub>6</sub> H <sub>4</sub> NH <sub>2</sub>	5	10
14	4-4'-Diaminodiphenylsulfoxide	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> SO C <sub>6</sub> H <sub>4</sub> NH <sub>2</sub>	1	3
15	4-4'-Diaminodiphenylsulfone <sup>5</sup>	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> SO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> NH <sub>2</sub>	1	2
16	4-4'-Diacetylaminodiphenylsulfone <sup>6</sup>	CH <sub>3</sub> CO NH C <sub>6</sub> H <sub>4</sub> SO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> NH <sub>2</sub>	10	
17	4-Nitro-4'-acetylaminodiphenyl sulfone (25)	NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> SO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> NH <sub>2</sub> COCH <sub>3</sub>	10	20
18	Promin <sup>7</sup>	[CH <sub>3</sub> OH(CHOH) <sub>4</sub> CH <sub>2</sub> SO <sub>3</sub> Na NH C <sub>6</sub> H <sub>4</sub> ] <sub>2</sub> SO <sub>3</sub>	5	20
19	Phosphanilic acid (26)	H <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> PO(OH) <sub>2</sub>	2	10
20	Dimethylaminophenylphosphonous acid (27)	(CH <sub>3</sub> ) <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> P(OH) <sub>2</sub>	200	
21	<i>Bis</i> -(dimethylaminophenyl) phosphinic acid (27)	[(CH <sub>3</sub> ) <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> ] <sub>2</sub> P(OH) <sub>2</sub>	20	
22	<i>Bis</i> -(dimethylaminophenyl) phosphinic acid <sup>8</sup>	[(CH <sub>3</sub> ) <sub>2</sub> N C <sub>6</sub> H <sub>4</sub> ] <sub>2</sub> PO(OH) <sub>2</sub>	10	50
23	<i>Bis</i> -(4'-aminophenyl) phosphinic acid <sup>9</sup>	[NH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> ] <sub>2</sub> PO(OH) <sub>2</sub>	100	

\* Compounds 1, 8, 9, 19, 20, 22, and 23 were used as the sodium salt after careful neutralization with NaOH.

<sup>1</sup> Prepared by Dr. E. L. Jackson of this laboratory.

<sup>2</sup> Prepared by Dr. H. Bauer of this laboratory by the method of Bamberger and Pyman (24).

<sup>3</sup> Courtesy of the Calco Chemical Co.

<sup>4</sup> Courtesy of the Burroughs Welcome Co.

<sup>5</sup> Prepared by Dr. H. Bauer.

<sup>6</sup> Courtesy of Parke, Davis & Co.

<sup>7</sup> Prepared by Dr. H. Bauer by the method of Bourneuf (28).

per cent was obtained with 4-4'-diaminodiphenylsulfide and sodium sulfadiazine. The most actively inhibiting compounds were sulfathiazole, which gave good in-

hibition at 5 mgm. per cent, 4-4'-diaminodiphenylsulfoxide at 3 mgm. per cent, and 4-4'-diaminodiphenylsulfone, which inhibited growth at a level of 2 mgm. per cent. Since promin is theoretically about 30 per cent diaminodiphenylsulfone, it is inferior in its tuberculostatic action compared with the parent substance. Substitutions in the amino groups by acetylation or by replacement with nitro groups reduced inhibiting action, while oxidation to the hydroxylamine seems to have had little or no effect. Substitution in the sulfonamide group has resulted in increased activity as in sulfapyridine, sulfathiazole, and sulfadiazine, or in decreased activity as in the instance of sulfanilylaminooethanol.

In the small series of phosphorus compounds phosphanilic acid was the most inhibiting, comparing in activity with 4-4'-diaminodiphenylsulfide and sulfadiazine. This would appear to be interesting for the corresponding sulfur analog, sulfanilic acid, was inactive up to 500 mgm. per cent. It seems probable that with phosphanilic acid as a starting point it may be possible to develop compounds more active than the corresponding sulfur analogs. However, an attempt in this direction represented by compounds 20, 21, 22, and 23 listed in Table 1 failed to attain the goal though, as will be pointed out later, compound No. 21 does appear to have a retarding influence *in vivo* if not *in vitro*. This, we suspect, is due to demethylation in the body to the free amino compound. The lack of activity *in vitro* of compound No. 23, which has free amino groups, does not exclude the possibility that the corresponding trivalent phosphorus compound may prove active. Efforts to prepare this have so far been unsuccessful.

**THERAPEUTIC TESTS.** From the foregoing list compounds 2, 10, 11, 12, 15, 18, 19, and 21 were selected for therapeutic tests in guinea pigs infected with two strains of human tubercle bacilli and in rabbits infected with a bovine strain.

In the first experiment guinea pigs weighing about 300 grams were divided into five groups each containing twelve animals; all were inoculated intraperitoneally with 0.5 mgm. *H* 57. For a week prior to inoculation the animals were kept on a diet consisting of 69 per cent ground rolled oats, 25 per cent ground alfalfa, 5 per cent casein, and 1 per cent sodium chloride (29). In addition 25 grams of carrots were allowed per animal per day. Immediately after inoculation the drugs were incorporated in this diet at a level of 0.5 per cent. A record kept of the amount of food consumed indicated an estimated drug intake of from 50 to 75 mgm. per day or about 150 to 300 mgm. per kilogram per day. The experiment lasted 56 days. Whenever an animal died in one of the groups, one animal appearing the sickest in each of the other groups was killed for comparison as to the extent of tuberculous involvement in the several groups. The extent of infection was rated from 0 to 4+ as follows:

+ = Slight. Few miliary tubercles of spleen, liver, omentum, or lungs.

++ = Moderate. Many discrete tubercles in any one of the foregoing organs.

+++ = Moderately advanced. Generalized miliary and conglomerate tubercles in one or more of the organs.

++++ = Advanced caseation necrosis of spleen, liver, omentum, lungs, or all of these.

This strain proved to be of low virulence, the extent of involvement seldom exceeding 2 or 3+, and usually not more than 1+. The infection was usually limited to the omentum, glands, and spleen. From one to six animals died in each group within 25 days subsequent to infection and these are not included in the final analysis. Among those surviving 25 to 56 days, the results were as shown in table 2.

The numerical values for the extent of tuberculous involvement given in the third column

of this table were arrived at by dividing the sum of all the ratings by the number of animals autopsied in each group between the 25th day of the infection and the end of the experiment.

The results of this experiment (table 2) indicate that the drugs were sufficiently toxic to contribute in some measure to the mortality rate, and that none of them in the doses given, except possibly the last-named one, had any beneficial influence in retarding the tuberculous process. There was very little involvement in this group except in the spleens, which were very large, with little or no gross evidence of tubercle formation. Microscopically they usually presented nothing more than epithelioid aggregates or tubercles in early formation with little or no necrosis.

Towards the end of the experiment blood analyses were made in several animals in each group, the determinations having been made in terms of sulfanilamide in each case.<sup>1</sup> As seen in the last column of table 2 this was quite variable and

TABLE 2

*Effect of sulfanilamide and related compounds on experimental tuberculous infection in guinea pigs when fed at a level of 0.5 per cent in the diet*

GROUP	MORTALITY	AVERAGE EXTENT OF TUBERCULOSIS	BLOOD LEVELS	
			Free mgm. per cent	Total mgm. per cent
Controls.....	0/10 (0%)	1.4		
Sulfanilamide.....	5/11 (45%)	1.4	Trace-2.3	0.5-6.0
Sulfapyridine.....	8/11 (72%)	1.8	0.4-2.3	1.0-3.5
Sulfathiazole .....	4/6 (66%)	1.7	Trace, 0.4	0.4-0.8
<i>Bis</i> -(dimethylaminophenyl) phosphorous acid...	2/10 (20%)	0.3	Trace-0.9	0.5-1.0

generally low. It was obviously difficult by this method of administration to maintain a uniform level of drug intake.

In the second experiment a more virulent strain of tubercle bacilli was used, the animals were kept on a more satisfactory diet of Purina rabbit chow supplemented with cabbage three times a week to supply adequate vitamin C (in which the Purina chow is lacking) and the drugs were administered intragastrically once daily, except Sundays. A No. 8 silk ureteral catheter connected to a syringe was used as a stomach tube. The water-soluble promin and sodium salt of phosphanilic acid were given in aqueous solution, all the others in aqueous suspension with five per cent gum acacia.

In this series there were 96 animals, 16 to a group. The first group served as controls, the second received phosphanilic acid as the sodium salt, the third *bis*(dimethyl amino phenyl) phosphorous acid, the fourth promin, the fifth diaminodiphenylsulfone, and the sixth sulfadiazine. Promin, sulfadiazine, and phosphanilic acid were given in doses of 0.5 gram per kilogram. This dosage was well tolerated. Diaminodiphenylsulfone was

<sup>1</sup> The method of Bratton and Marshall (J. Biol. Chem., 128: 537, 1939) was used.

given in 0.1 to 0.15 gram per kilogram, and *bis*(dimethyl amino phenyl) phosphinous acid in doses of from 0.2 to 0.5, usually 0.3 gram per kilogram. It seemed desirable to give these drugs to the limits of tolerance. The minimum lethal dose of diaminodiphenylsulfone in guinea pigs on repeated administration was found to be 0.25 gram per kilogram, that of *bis*(dimethyl amino phenyl) phosphinous acid was more variable, the toxic range being between 0.3 and 0.5 gram per kilogram. This drug is more cumulative and less certain in its action. Though a single dose of 0.7 gram per kilogram is usually survived, 0.3 gram per kilogram may prove fatal after repeated daily administrations.

Treatment in this series of experiments was continued for one month. Since all the evidence indicates that the beneficial action of these drugs, if any, is due to inhibition of the multiplication of the tubercle bacillus in the body and a retardation of the tuberculous process possibly through attenuation, it seemed unnecessary to continue treatment beyond the time when dissemination of the tuberculous process is normally well under way. Rather it seemed desirable to give as intensive treatment as possible during the early period of infection.

Accordingly, the animals were infected intraperitoneally with a heavy dose, 0.5 mgm. moist weight, of a virulent human strain of tubercle bacilli A 27.<sup>2</sup> Treatment was begun on the same day and continued for one month. The animals were weighed once a week and observed carefully for symptoms. At the death of an animal, or when this appeared imminent, the animal was killed and autopsied. A system of rating of the extent of tuberculous involvement, different from that described in the first experiment, was adopted. This consisted in rating the four most commonly involved organs, omentum and glands, spleen, liver, and lungs, from 0 to 4+. One plus designated slight involvement, a few miliary pin point tubercles, 2+ moderate miliary dissemination, 3+ generalized miliary and conglomerate dissemination with occasional patchy caseous necrosis, and 4+ extensive involvement with much caseous necrosis. The sum of these divided by the number of organs gave a numerical value of the extent of tuberculous involvement for each animal. At the end of 107 days when 81 per cent of the controls and 94 per cent in one of the treated groups had died, all the survivors were injected subcutaneously with 0.1 mgm. tuberculo protein (P.P.D.).<sup>3</sup> After 24 hours all the survivors were killed and the experiment terminated.

The results of this experiment are summarized in table 3. The comparative mortality rate for the several groups is given at ten-day intervals, and the extent of tuberculous involvement for each animal in each of the groups as well as the average for the whole group is expressed numerically. Examination of the data reveals no favorable effects in the group treated with phosphanilic acid, a definitely retarding influence on the dissemination of the tuberculous process in the group treated with *bis*-(dimethyl amino phenyl) phosphinous acid, and a distinctly favorable influence, both as regards the dissemination of the tuberculous process as well as the survival time, in the groups treated with promin, sulfadiazine and diaminodiphenylsulfone. The last named drug has, on the whole, appeared to be the most effective. This is indicated by the lowest tuberculosis index and the largest number of survivals following the subcutaneous injection of tuberculin. A comparison of the number of animals showing very slight or no gross tuberculous involvement in these three groups indicates one in the promin series, four in the sulfadiazine, and five in the diaminodiphenylsulfone series.

<sup>2</sup> This strain was obtained from the Henry Phipps Institute of Philadelphia, through the courtesy of Doctor F. Seibert.

<sup>3</sup> Courtesy of Doctor F. Seibert, Henry Phipps Institute of Philadelphia.

TABLE 3  
Mortality rate and extent of tuberculous involvement at 10-day intervals

DATE	CONTROLS		PHOSPHANILIC ACID		BIS(DIMETHYLAMINOPHENYL)PHOSPHINOUS ACID		PROMIN		DIAMINO-DIPHENYL-SULFONE		SULFADIAZINE	
	Per cent mortality	Tuberculosis	Per cent mortality	Tuberculosis	Per cent mortality	Tuberculosis	Per cent mortality	Tuberculosis	Per cent mortality	Tuberculosis	Per cent mortality	Tuberculosis
30-39	6	2.5	18	2.0 0.5 1.4	6	0.5	6	1.0	5	2.0	6	1.0
40-49	12	3.0	44	1.7 1.0 3.0 1.7	12	0.5	6		12	1.5	12	1.7
50-59	32	2.2 3.0 1.5	66	2.5 3.0	44	1.0 1.2 1.2 1.5 1.3	12	1.0	25	1.5 1.0	25	2.0 2.2
60-69	50	1.7 2.5 2.0	87	2.5 3.5 2.8 3.2 2.0	56	2.0 1.0	25	0.7 1.0	32	2.2	32	1.2
70-79	69	1.5 1.0 2.0	87		69	2.0 2.4	32	0.7	32		38	1.7
80-89	81	2.2 3.4	94	2.2	60		33	1.0	50	1.0 1.2 1.0	44	2.2
90-99	81		94		81	0.8 1.0	44	1.5	56	±	56	± 1.0*
107	81	3.2 2.5 2.0	94	2.0	81	1.7 1.5 1.0	44	1.2 0.2† 1.7	56	0.7 0.7 0.2† 1.5† 1.7 2.0 2.0 2.7 1.7	56	0.5† ±† ±† 1.7 0.7 3.0 2.0
Average tuberculosis index ...			2.3		2.3	1.3		1.4		0.9		1.4

\* Paralysis, killed.

† Survived 0.1 mgm P.P.D. subcutaneously.

More than half of the animals in the last-named group presented an uninterrupted growth curve after cessation of treatment, indicating attenuation of the tubercle bacillus or arrest of the infection.

At from 30 to 60 days, after treatment was discontinued, paralysis of the posterior extremities developed in each of two animals in the promin and sulfadiazine groups and in three animals in the diaminodiphenylsulfone group. It was suspected that the drugs were responsible for what seemed to be a peripheral neuritis. However, careful histological examination of the sciatic nerve and sections of the spinal cord failed to show any lesions. In several of the animals tuberculous infection was found in the meninges in the lumbar region of the spinal cord. It would appear that the paralyses were actually due to tuberculous meningitis. Whether the invasion of the central nervous system is a coincidence, or is in some manner related to treatment, is not possible to say.<sup>4</sup>

TABLE 4  
Blood levels, mgm. per cent  
(F = free, T = total)

HOURS	PHOSPHANILIC ACID		Bis(DIMETHYLAMINO- PHENYL) PHOS- PHINOUS ACID*		PROMIN†		DIAMINODIPHENYL- SULFONE		SULFADIAZINE	
	F	T	F	T	F	T	F	T	F	T
3	0.9	0.9	2.5	2.5	10.5 (3.4)	11.0 (3.5)	4.0	5.5	16.5	15.5
6	0.8	0.8	3.0	3.0	13.5 (4.3)	14.0 (4.3)	4.5	5.0	16.5	17.0
24	0.8	0.8	0.1	0.1	8.0 (2.6)	8.0 (2.6)	3.5	4.5	6.5	6.5

\* Estimations made in terms of sulfanilamide, the latter having been used as standard.

† Figures in parentheses are diaminodiphenylsulfone equivalents.

In an attempt to correlate the therapeutic effectiveness of the foregoing drugs with their concentration in the blood a series of normal guinea pigs were treated in the same manner as the infected animals for a period of from 6 to 13 days and the blood levels determined at intervals of from 3 to 24 hours following the last dose. The results of this experiment, expressed as averages of 3 to 4 animals, are shown in table 4. If the drug has some specificity of action, as would appear from its tuberculostatic action *in vitro*, the blood concentration of phosphanilic acid was probably too low to be effective. This and similar experiments in rabbits have shown the poor absorbability of this drug from the gastrointestinal canal. The second phosphorus compound in this series, bis (dimethyl amino phenyl) phosphinous acid, is absorbed with sufficient regularity, and is apparently demethylated to a sufficient degree, to yield a fairly good blood level which is not, however, well maintained despite its cumulative action judged on

<sup>4</sup> We are indebted to Doctor R. D. Lillie of the Division of Pathology for the histological examination of the tissues.

the basis of toxicological action. The blood level of promin was fairly uniform and well maintained, though perhaps it was not as high as that of diaminodiphenylsulfone when calculated in terms of its equivalent. The somewhat higher and more uniform blood level of diaminodiphenylsulfone, together with its stronger bacteriostatic action *in vitro*, may account for its superior therapeutic effectiveness compared with promin. Under the experimental conditions of treatment the blood level of sulfadiazine was the highest, though it was not as uniform throughout the 24 hours as was the case with the other sulfur compounds. Attention should be called to the low rate of conjugation of this drug compared to the well known high rate of conjugation of sulfanilamide in the guinea pig. This confirms the experiments of Feinstone and associates (30). The lower therapeutic effectiveness of sulfadiazine, despite its higher blood levels compared with diaminodiphenylsulfone, could be explained on the basis of lower specificity as indicated by the relative tuberculostatic actions of these drugs in Table 1.

Two series of experiments with some of the foregoing drugs were also carried out in rabbits.

In these a highly virulent bovine strain of tubercle bacilli, *Ravenel* (S), was used. In the first series 18 rabbits, weighing 2.0 to 3.5 kgm., were each inoculated intravenously with 0.016 mgm. of a fine suspension of tubercle bacilli in 1 cc. sterile salt solution. Six animals served as controls, 6 were treated daily with 0.1 gram per kilogram sodium sulfathiazole and 6 with 0.1 gram per kilogram phosphanilic acid as the sodium salt, both given intravenously. At death the animals were autopsied and the extent of tuberculous involvement noted. This was rated from 0 to 4+ in each of the four organs showing macroscopic lesions, lungs, liver, kidneys, and spleen. The sum of these divided by four gave the "tuberculosis index" for each of the animals, and the sum of these indices divided by the number of animals in the group gave the average index for the group. In the second series of experiments 16 rabbits weighing from 2.0 to 3.0 kgm. were inoculated intravenously with 0.015 mgm. of tubercle bacilli as above. Six served as controls and 10 were treated intravenously daily with 0.3 to 0.5 gram of promin per kilogram, in 10 per cent aqueous solution. Treatment was continued until death, the longest period being 45 days when the last of the controls died. The drug was given in maximum tolerated doses. Doses of 0.3 gram per kilogram usually produced no noticeable effects, while doses of 0.4 and 0.5 gram per kilogram often produced distressing symptoms of dyspnea. Survival time and post mortem findings were noted as in the preceding series.

The results of this study are summarized in tables 5 and 6. Sulfathiazole appeared to inhibit the dissemination of the tuberculous process but had no favorable effect on the survival time. Phosphanilic acid had a more favorable influence. This drug, however, leaves the blood stream rather rapidly, hence it is not possible to maintain an effective blood level with a single daily injection. The rapidity with which the drug leaves the blood stream when injected intravenously or subcutaneously is shown in table 7. The excretion of phosphanilic acid begins soon after injection. The urine turns acid and crystals, apparently of the insoluble acid, appear in abundance. Eighty to 90 per cent of the amount injected is eliminated in the urine within 24 hours, with practically no conjugation.

The toxicity of this compound is low. Rats tolerated one gram per kilogram

TABLE 5  
*Effect of sulfathiazole and phosphanilic acid in bovine tuberculosis in rabbits*

NUMBER	CONTROLS			SODIUM SULFATHIAZOLE			PHOSPHANILIC ACID		
	Weight	Days	Tuberculosis index	Weight	Days	Tuberculosis index	Weight	Days	Tuberculosis index
1	2.0	36	2.5	3.0	19	0.5	2.6	36	1.2
2	3.0	40	2.5	2.4	40	1.5	2.8	46	2.8
3	3.4	46	2.8	2.5	40	2.2	2.9	53	2.0
4	2.2	47	2.3	2.5	49	2.0	2.5	57	1.2
5	2.4	50	2.2	3.7	53	2.5	2.9	77	1.5
6	4.0	53	2.2	3.3	70	2.0	3.1	97	1.3
Average survival...		45.3			45.3			61	
Average tuberculosi- s index.....			2.4			1.8			1.7

TABLE 6  
*Effect of promin in experimental bovine infection in rabbits*

NUMBER	CONTROLS			TREATED		
	Weight	Days	Tuberculosis index	Weight	Days	Tuberculosis index
1	2.1	21	0.8	2.1	16	0.5
2	2.2	24	2.0	2.1	19	1.2
3	2.3	30	2.5	3.0	24	1.2
4	2.4	31	2.7	2.0	25	1.5
5	2.2	33	2.8	2.5	30	1.2
6	2.3	45	1.5	2.0	30	2.5
7				2.3	39	1.5
8				2.2	53	1.7
9				2.5	74	1.5
10				2.2	122	1.0
Average survival.....		30.7			43.2	
Average tuberculosis index.....			2.1			1.4

TABLE 7  
*Fate of phosphanilic acid in the rabbit injected as the sodium salt*

TIME	INTRAVENOUS INJECTION, 0.3 GRAMS PER KGM.		SUBCUTANEOUS INJECTION, 0.5 GRAMS PER KGM.	
	Blood level			
	Free	Total	Free	Total
5 minutes	110.0	130.0		
1 hour	49.0	50.0	86.0	97.6
2 hours			61.5	86.1
3 hours	13.0	13.2		
4 hours			24.5	25.0
5 hours	4.0	4.2		
6 hours			6.3	6.1
24 hours	Trace	Trace	Trace	Trace

injected intravenously, and in an experiment in a cat under amytal anesthesia a slow intravenous infusion of 2.0 grams per kilogram as a 5 per cent solution of the sodium salt had no effect on the blood pressure or respiration.

The effects of promin seem definite. The survival period in the treated animals was longer, and the extent of tuberculous infection distinctly less. Tuberculous infection was present, however, in every animal; and since the dose used was near the toxic limit, it would seem that promin is less effective in infections with the bovine strain of tubercle bacilli than with the human.

**COMMENT.** It has been possible to retard the tuberculous process or to check the progress of the disease in experimental animals by means of some of the sulfonamides, sulfones, and certain of the related phosphorus compounds. Rich and Follis (1) were able to do this with sulfanilamide which, however, required toxic doses to achieve the result. In the present study it was possible to accomplish similar results with doses of drugs well within the tolerated range. This suggests greater specificity of some of the drugs we have used. The present experiments also indicate a close parallelism between the effects of the drugs we have used in experimental animals and their tuberculostatic action *in vitro*. All the experiments taken together strongly indicate that diaminodiphenylsulfone is the most effective agent, both *in vitro* and *in vivo*. The toxic nature of this compound and the extreme care with which it has to be administered, coupled with its high degree of specificity, make the search for more effective and less toxic derivatives a promising field of investigation. Diaminodiphenylsulfone appears to have a definitely retarding influence when administered at a level of 50 to 75 per cent of the lethal dose. With the use of this drug as a standard for comparison, the obvious aim is to develop derivatives capable of producing the same or better effects with dosages further removed from the toxic level. Promin does not fulfill this requirement, though it appears to be a step in the right direction.

#### SUMMARY

A series of sulfonamides, sulfones, and certain related phosphorus compounds were examined for tuberculostatic action *in vitro*. Good inhibition in decreasing order of magnitude was obtained with diaminodiphenylsulfone, diaminodiphenylsulfoxide, sulfathiazole, diaminodiphenylsulfide, sulfadiazine, phosphanilic acid, and promin. Therapeutic tests in experimental animals showed a favorable effect both as regards survival time and retardation of the progress of the disease with diaminodiphenylsulfone, promin, and sulfadiazine. A doubtful effect was obtained with sulfathiazole and *bis* (dimethyl amino phenyl) phosphorous acid, and an irregular result with phosphanilic acid. The last-named drug is poorly absorbed from the gastrointestinal tract, and it leaves the blood stream rather rapidly on intravenous or subcutaneous injection, making the maintenance of a satisfactory blood level difficult.

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## STUDIES ON SULFONAMIDE-RESISTANT ORGANISMS

### I. DEVELOPMENT OF SULFAPYRIDINE RESISTANCE BY PNEUMOCOCCI

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In our earlier experiments (1, 2) it was noted that under certain conditions sulfanilamide and sulfapyridine inhibited growth of pneumococci for a limited time only. Thus, in *in vivo* experiments (2) in which infected mice were treated with less than curative doses of sulfapyridine, the number of pneumococci in the blood remained stationary at a low level during the first four or five days of treatment; then, notwithstanding continued therapy, the number of organisms increased rapidly and the infection quickly overwhelmed the animals. Observations similar to these were made in *in vitro* experiments (2), in which the growth of pneumococci in sulfapyridine-containing broth was being studied. In these experiments the period of growth inhibition terminated after 24 to 60 hours of incubation. Since this loss in effectiveness of sulfapyridine was not due to a decrease in concentration of the drug, either in the culture medium or the infected animal, it seemed likely that the pneumococci had in some manner adapted themselves to growth in the presence of this drug; in other words, they had become sulfapyridine-resistant or sulfapyridine-fast.

At the time these observations were made there was little information on the phenomenon of sulfonamide resistance. MacLean, Rogers and Fleming (3) had reported that pneumococci acquired resistance to sulfapyridine *in vivo*, but the observed changes in sensitivity were relatively small and not entirely conclusive. MacLeod and Daddi (4) reported development of a sulfapyridine-resistant strain of pneumococcus by an *in vitro* technique, but this report was preliminary and gave no details as to the rate at which resistance developed. In view of the practical and theoretical implications of these observations on sulfapyridine resistance, a more complete and critical study seemed indicated. Such a study was undertaken with attention being paid to the conditions of the *in vivo* and *in vitro* experiments mentioned above. Preliminary reports of this work have been presented heretofore (5, 6); the detailed results are presented here.<sup>1</sup>

<sup>1</sup> Since the preliminary reports of this work were presented, Schmitt (7) and Lowell, Strauss and Finland (8) published the results of their *in vitro* studies on the development of sulfonamide resistance by pneumococci. The *in vivo* development of such resistance has been reported by Mulder (9) and Schmitt (10). Whereas the resistance developed in the experiments of the above workers was not as great as that produced in our experiments, the essential findings in the above publications agree with those reported here.

DEVELOPMENT OF RESISTANCE *in vivo*

*A. Method.* One strain of type I pneumococcus and two strains of type III were used in this experiment. These strains had been used in our previous studies; consequently, their responses to sulfapyridine were fairly well established. Stock cultures of these organisms were passed repeatedly through mice until constant virulence and invasiveness were attained. Then the organisms were passed serially through groups of sulfapyridine-treated mice in the following manner. Thirty to forty white mice, males, weighing 18 to 22 grams, were infected intraperitoneally, each mouse receiving  $10^{-6}$  cc. of a 12- to 14-hour blood broth culture of the desired organism. Ten of these mice were kept as untreated controls. The remaining animals were treated with sulfapyridine. In the initial experiments, the mice infected with the type I strain received 5 mgm. doses of sulfapyridine, whereas those infected with the type III strains received 20 mgm. doses. These amounts, suspended in 10 per cent acacia, were administered by stomach tube at 2, 8, 14, and 22 hours after infection and every 8 hours thereafter for five additional days. These treatments were based on earlier experiments which showed that 5 mgm. doses in infections with the type I strain and 20 mgm. doses in infections with the type III strains prolonged life for at least six days, but cured no more than one-third of the infected mice.

Cultures of heart blood in infusion broth were prepared from at least 6 treated mice that died between seven and ten days after infection. These cultures were incubated at 37.5°C. for 12 to 14 hours and then pooled; 0.5 cc. of this pooled culture was injected intraperitoneally into an untreated mouse. A 12- to 14-hour blood-broth culture prepared from the heart blood of this mouse was used to infect a second group of mice which received the same sulfapyridine treatment as the first group.

This passage procedure was repeated until the survival time of the treated mice was reduced to such an extent that it approximated the survival time of the untreated controls. Experiments with the type III strains, CHA and Wistuba, were terminated at this point, since in the initial experiments 20 mgm. doses of sulfapyridine were administered. In the experiments with the type I strain, McGovern, where 5 mgm. doses were used initially, additional passages were carried out with the sulfapyridine dosage increased first to 10 and then to 20 mgm. No doses larger than 20 mgm. were used since previous experiments showed that this dosage of sulfapyridine administered at 8-hour intervals had as much curative action as larger doses.

At least two serial experiments, such as the above, were carried out with each of the three strains mentioned. The essential findings in duplicate experiments were identical. The results of typical experiments with each strain have been summarized in tables 1, 2, and 3.

*B. Results.* The type III strains, CHA and Wistuba, which were naturally slightly resistant to sulfapyridine *in vivo*, acquired a high degree of resistance after as few as 3 serial passages. As table 1 shows, the resistance of strain CHA was increased markedly by the first passage. Thus, mice infected with organisms that had been passed once through sulfapyridine-treated animals (Experiment B) lived on the average only 81 hours, whereas those infected with the parent strain (Experiment A) lived 185 hours. The second and third passages produced a further increase in resistance, so that mice infected with organisms passed three times through sulfapyridine-treated animals (Experiment D) lived only 46 hours—i.e., just 20 hours longer than the untreated controls. This represented the limit of resistance that could be obtained using the procedure described, since three additional passages failed to increase resistance to the drug (cf. Experiments E, F, and G).

In contrast to the result with strain CHA, the first serial passage produced no apparent change in the sensitivity of strain Wistuba (cf. Experiments A and B, table 2). The second passage, however, led to an enormous increase in resist-

TABLE 1

*Development of sulfapyridine resistance by type III pneumococcus, strain CHA*

EX- PER- IMENT	ORGANISMS		NUM- BER OF MICE IN- FECTED	TREAT- MENT	NUMBER OF DEATHS							SUR- VIVAL OF MICE THAT DIED hours	THIRTY-DAY SURVIVORS				
	Source	Number in infecting dose			Days after infection:								Num- ber	Per cent			
					1	2	3	4	5	6	7-10						
A	From stock after 85 passages	600	30	SP20*	0	0	0	0	2	5	23	185	0	0			
			10	None	7	3	0	0	0	0	0	24	0	0			
B	From 6 exper. A mice, dead on day 7	330	27	SP20	0	1	9	13	2	2	0	81	0	0			
			10	None	10	0	0	0	0	0	0	22	0	0			
C	From 6 exper. B mice, dead on day 3	300	30	SP20	0	5	20	5	0	0	0	59	0	0			
			10	None	8	2	0	0	0	0	0	24	0	0			
D	From 10 exper. C mice, dead on day 3	250	30	SP20	0	22	8	0	0	0	0	40	0	0			
			10	None	4	6	0	0	0	0	0	26	0	0			
E	From 10 exper. D mice, dead on day 2	400	30	SP20	0	21	9	0	0	0	0	43	0	0			
			10	None	7	3	0	0	0	0	0	23	0	0			
F	From 11 exper. E mice, dead on day 2	300	30	SP20	0	20	10	0	0	0	0	45	0	0			
			10	None	4	6	0	0	0	0	0	26	0	0			
G	From 8 exper. F mice, dead on day 2	300	30	SP20	0	19	10	1	0	0	0	45	0	0			
			10	None	7	3	0	0	0	0	0	24	0	0			
H	From stock after 109 passages	300	30	SP20	0	0	0	0	1	0	29	186	0	0			
			10	None	6	4	0	0	0	0	0	24	0	0			

\* SP20 = 20 mgm. sulfapyridine 2, 8, 14, and 22 hours after infection and every 8 hours thereafter for 5 days, or as long as the animals survive.

ance; thus when mice were infected with pneumococci that had been passed twice through treated animals (Experiment C), their average survival time was only 58 hours, as compared with survival times of 173 and 171 hours for mice infected with the parent strain and the organisms recovered from the first serial passage (Experiments A and B). As Experiment D shows, the third serial

passage led to a further increase in the resistance of strain Wistuba. As in the experiment with strain CHA, however, no greater resistance was acquired during two additional passages (Experiments E and F).

More serial passages were required to make type I, strain McGovern, highly resistant than were necessary in the experiments with the type III organisms. This was probably due to the fact that this type I strain was originally much more sensitive to sulfapyridine than either type III strain. However, after 9 serial passages through groups of mice treated with 5, then 10, and finally 20 mgm. doses of sulfapyridine, strain McGovern was as resistant to this drug as were strains CHA and Wistuba (table 3). Comparison of Experiments A and J shows the striking difference in the response of strain McGovern before and after passage through sulfapyridine-treated animals. Thus of the mice infected with the parent strain (Experiment A), 40 per cent of those receiving 5 mgm. doses of sulfapyridine and 83 per cent of those receiving 20 mgm. doses recovered from the infection; the average survival times of the mice that did not recover were 170 and 234 hours for the respective groups. In contrast to this, 5, 10, and 20 mgm. doses of sulfapyridine were entirely without curative action against infections with organisms isolated from the ninth serial passage (Experiment J). In fact, the average survival time of the treated animals, 50 hours, was only 18 hours more than that of the control animals.

It is noteworthy that the resistance of strain McGovern to increasing doses of sulfapyridine developed progressively (cf. Experiments A to I). Nevertheless, when resistance to 20 mgm. doses of this drug was well established, 5 and 10 mgm. doses prolonged life as much as 20 mgm. doses (Experiment J). The explanation for this fact is not clear as yet.

It is particularly important to note the final experiments in tables 1, 2, and 3. These experiments were designed to determine the effect of animal passage on the response to sulfapyridine. They were carried out in the same manner as the first experiment in each table, except that the infecting organisms were the parent organisms that had been passed through untreated mice once daily during the time required to make the different strains resistant. Comparison of the results of these final experiments with those of Experiment A of each table shows conclusively that continued animal passage *per se* was not responsible for the changes in resistance described above.

*C. Comparison of the *in vitro* response of the parent and resistant strains.* Since it has been observed (2, 11, 12) that the response of pneumococci *in vivo* does not necessarily parallel their response *in vitro*, it seemed of interest to know whether these organisms that had acquired resistance to sulfapyridine in the preceding *in vivo* experiments were likewise resistant to this drug *in vitro*. Accordingly, a comparative study was made of the growth of the parent and resistant organisms in media containing various concentrations of sulfapyridine.

The medium selected for this work was a beef heart infusion broth (2) enriched with 3 per cent rabbit blood; 50 cc. quantities of this medium, containing 0, 10, and 20 mgm. per cent sulfapyridine, were placed in milk dilution bottles, then

inoculated with 0.5 cc. of a  $10^{-4}$  dilution of a 12-hour culture of the desired organism and incubated at 37.5°C. At various intervals, 0.5 cc. samples of culture were withdrawn, diluted serially and the numbers of organisms in appropriate dilutions were determined by means of blood agar pour plates.

TABLE 2

*Development of sulfapyridine resistance by type III pneumococcus, strain wistuba*

EX- PERI- MENT	ORGANISMS		TREAT- MENT	NUMBER OF DEATHS							SUR- VIVAL OF MICE THAT DIED	THIRTY-DAY SURVIVORS			
	Source	Num- ber in in- fecting dose		Days after infection:								Num- ber	Per cent		
				1	2	3	4	5	6	7-10					
A	From stock after 156 passages	500	20	SP20*	0	0	0	0	0	1	15	173	4	20	
			10	None	7	3	0	0	0	0	0	25	0	0	
B	From 7 exper. A mice, dead on day 8	560	30	SP20	0	0	0	0	0	1	28	171	1	3	
			10	None	5	5	0	0	0	0	0	25	0	0	
C	From 6 exper. B mice, dead on day 7	900	30	SP20	0	4	24	1	0	1	0	58	0	0	
			10	None	8	2	0	0	0	0	0	25	0	0	
D	From 8 exper. C mice, dead on day 3	280	30	SP20	0	24	6	0	0	0	0	43	0	0	
			10	None	8	2	0	0	0	0	0	22	0	0	
E	From 9 exper. D mice, dead on day 2	890	30	SP20	0	16	13	1	0	0	0	40	0	0	
			10	None	6	4	0	0	0	0	0	23	0	0	
F	From 6 exper. E mice, dead on day 2	800	30	SP20	0	21	9	0	0	0	0	44	0	0	
			10	None	6	4	0	0	0	0	0	25	0	0	
G	From stock after 192 passages	500	30	SP20	0	0	0	0	2	1	17	166	10	33	
			10	None	5	5	0	0	0	0	0	25	0	0	

\* SP20 = 20 mgm. sulfapyridine 2, 8, 14, and 22 hours after infection and every 8 hours thereafter for 5 days, or as long as the animals survive.

The results of typical experiments are shown in figure 1. These data show that the resistant strains of type I McGovern and type III Wistuba multiplied as rapidly in broth containing 20 mgm. per cent sulfapyridine as in control broth. Growth of the resistant strain of type III CHA was inhibited slightly by 20 mgm. per cent sulfapyridine, but not at all by 10 mgm. per cent. Contrasted with this are the results with the parent organisms, growth of which was in-

TABLE 3

Development of sulfapyridine resistance by type I pneumococcus, strain McGovern

EX- PER- IMENT	ORGANISMS		NUM- BER OF MICE IN- FECTED	TREAT- MENT	NUMBER OF DEATHS						SUR- VIVAL OF MICE THAT DIED	THIRTY-DAY SURVIVORS			
	Source	Num- ber in infect- ing dose			Days after infection:							Num- ber	Per cent		
					1	2	3	4	5	6	7- 10				
A	From stock after 290 passages	380	30 30 10	SP5* SP20* None	0	0	0	0	1	8	9	170 234 30	12 25 0	40 83 0	
					0	0	0	0	0	0	5				
					1	9	0	0	0	0	0				
B	From 8 exper. A mice, dead on day 6	740	30 10	SP5 None	0	0	0	2	2	6	14	155 33	6 0	20 0	
					0	10	0	0	0	0	0				
C	From 8 exper. B mice, dead on day 3	680	30 10	SP5 None	0	2	16	7	2	3	0	72 28	0 0	0 0	
					0	10	0	0	0	0	0				
D	From 9 exper. C mice, dead on day 3	1280	30 10	SP10* None	0	1	9	4	7	1	5	98 37	3 0	10 0	
					0	10	0	0	0	0	0				
E	From 8 exper. D mice, dead on day 3	550	30 10	SP10 None	0	0	4	2	12	2	10	124 38	0 0	0 0	
					2	8	0	0	0	0	0				
F	From 9 exper. E mice, dead on day 5	1150	30 10	SP10 None	0	2	11	6	2	3	6	104 37	0 0	0 0	
					0	10	0	0	0	0	0				
G	From 8 exper. F mice, dead on day 3	550	30 10	SP10 None	0	18	10	2	0	0	0	53 35	0 0	0 0	
					0	10	0	0	0	0	0				
H	From 12 exper. G mice, dead on day 2	640	30 10	SP20 None	0	11	13	6	0	0	0	57 34	0 0	0 0	
					0	10	0	0	0	0	0				
I	From 12 exper. H mice, dead on day 2	520	30 10	SP20 None	0	5	23	2	0	0	0	58 38	0 0	0 0	
					0	10	0	0	0	0	0				
J	From 12 exper. I mice, dead on day 2	440	30 30 30 10	SP5 SP10 SP20 None	1	18	8	3	0	0	0	49 50 50 32	0 0 0 0	0 0 0 0	
					3	12	13	2	0	0	0				
					0	15	15	0	0	0	0				
					0	10	0	0	0	0	0				
K	From stock after 330 passages	450	30 30 30 10	SP5 SP10 SP20 None	0	0	0	0	0	2	8	171 174 189.5 36	20 26 28 0	67 87 93 0	
					0	0	0	0	2	0	2				
					0	10	0	0	0	0	0				
					0	10	0	0	0	0	0				
					0	10	0	0	0	0	0				

\* SP5, 10 and 20 = 5, 10 or 20 mgm. sulfapyridine 2, 8, 14, and 22 hours after infection and every 8 hours thereafter for 5 days, or as long as the animals survive.

bibited markedly by 10 mgm. per cent of the drug. It is evident, therefore, that these strains that had been made resistant *in vivo* were likewise resistant *in vitro*.

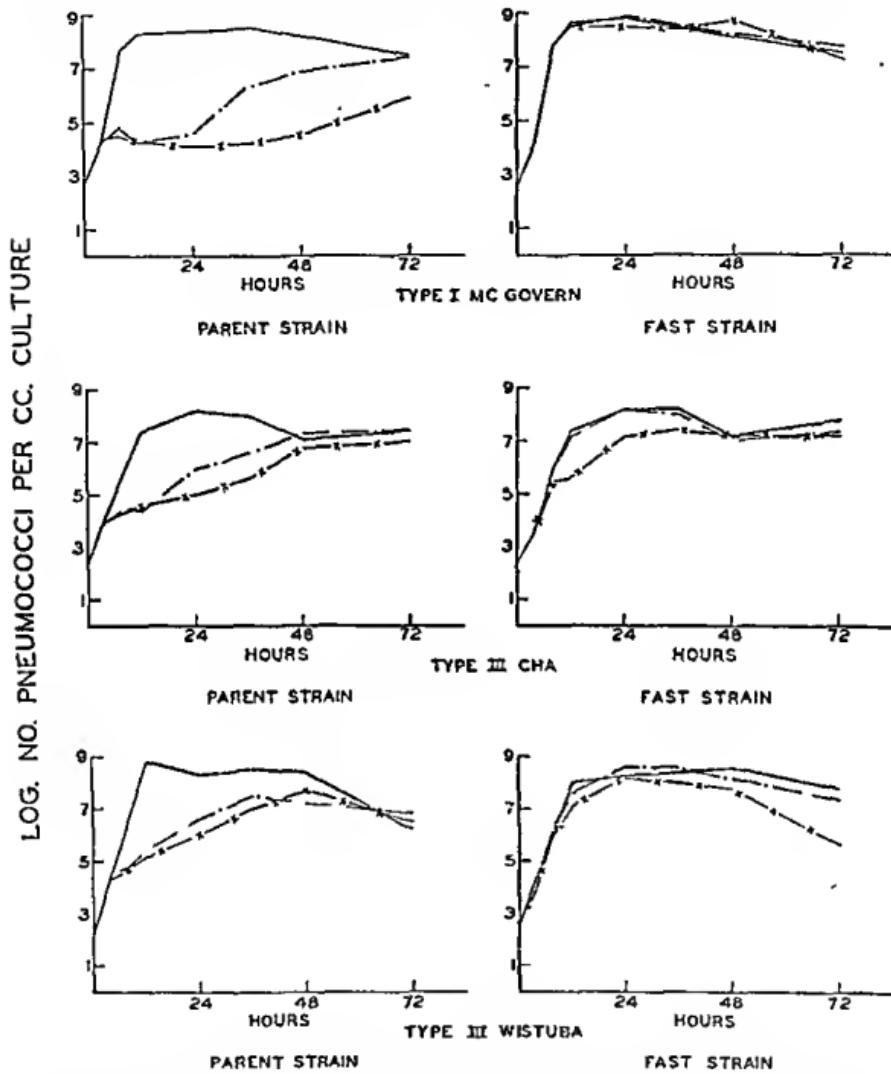


FIG. 1. EFFECT OF SULFAPYRIDINE ON GROWTH OF PARENT AND RESISTANT STRAINS OF TYPE I PNEUMOCOCCUS McGOVERN AND TYPE III PNEUMOCOCCI CHA AND WISTUBA

—, growth in beef heart control media; - - -, growth in beef heart media containing 10 mgm. per cent sulfapyridine; - - X - , growth in beef heart media containing 20 mgm. per cent sulfapyridine.

#### DEVELOPMENT OF RESISTANCE *in vitro*

**A. Method.** A type II pneumococcus, strain CH, was used in these experiments. This organism had undergone nearly 100 consecutive daily mouse passages prior to use and was selected for the present study on the basis of earlier experiments (2) which showed that it

was highly sensitive to sulfapyridine *in vitro*. The following procedure was used to make this organism highly resistant to the drug. Fifty cubic centimeter quantities of beef heart infusion broth, containing 0, 4, 8, and 15 mgm. per cent sulfapyridine, were placed in milk dilution bottles. Each quantity of medium was enriched with 1.5 cc. of rabbit blood, inoculated with 0.5 cc. of a  $10^{-4}$  dilution of a 12-hour blood broth culture of the parent organism, and then incubated at 37.5°C. At various intervals 0.5 cc. samples of the cultures were withdrawn, diluted serially, and the numbers of organisms in appropriate dilutions determined by means of blood agar pour plates. When growth had occurred in the culture containing 8 mgm. per cent sulfapyridine, the organisms from this culture were subcultured into plain beef heart infusion broth enriched with blood. This latter culture was incubated for 12 hours, then used as the source of organisms for a second experiment, otherwise identical with the first. This general procedure was repeated in 5 additional experiments; in these, however, the sulfapyridine content of the medium was increased gradually, until growth was obtained in media containing 160 mg. per cent of the drug ("saturated" media as shown in chart 7 of fig. 2).

In order to determine whether prolonged culture in artificial media in itself altered the sensitivity of strain CH to sulfapyridine, the following experiment was carried out simultaneously with the one just described. The parent organism was cultured in plain beef heart infusion broth, enriched with blood, for the same time and subcultured at the same intervals as the organisms that were transferred through sulfapyridine-containing broth. At the conclusion of these passages in plain broth, the sensitivity of this parent organism was studied, using a procedure identical with that described in the first test above.

*B. Results.* Charts 1 to 7 of figure 2 show graphically the changes in sensitivity which occurred as type II, strain CH, was passed serially through media containing increasing concentrations of sulfapyridine. The first experiment (Chart 1) shows the response of the parent organism. Growth of this organism was inhibited slightly in media containing 4 mgm. per cent sulfapyridine and was checked completely by 15 mgm. per cent of the drug. Growth in media containing 8 mgm. per cent sulfapyridine was inhibited almost completely for nearly 48 hours, then slow multiplication occurred. The result obtained after 6 serial passages of this organism through sulfapyridine-containing media is in marked contrast to that just described. After this number of passages (Chart 7), growth was nearly as rapid in media containing 90 and 160 mgm. per cent sulfapyridine as in control media. The results in Charts 2 to 6 indicate that this change in sensitivity was brought about progressively, with a small but definite increase in resistance occurring even during the initial passage through the sulfapyridine-containing media. The results shown in Charts 4 and 5 are particularly indicative of a progressive development of resistance. Thus, in the experiment shown in Chart 4, the concentrations of sulfapyridine were increased too much over those used previously, with the result that growth failed to occur in the presence of higher drug concentrations than those used in the preceding experiment. In the next experiment, however (Chart 5), the concentrations of sulfapyridine were raised more gradually, with the result that growth occurred in a higher concentration of drug than was used previously.

The results of the control experiment are shown in Chart 8 of figure 2. These data show conclusively that repeated culture of the parent organism in plain beef heart infusion broth was not in itself responsible for the increase in resistance noted above. In fact, the natural resistance of the parent organism seemed to

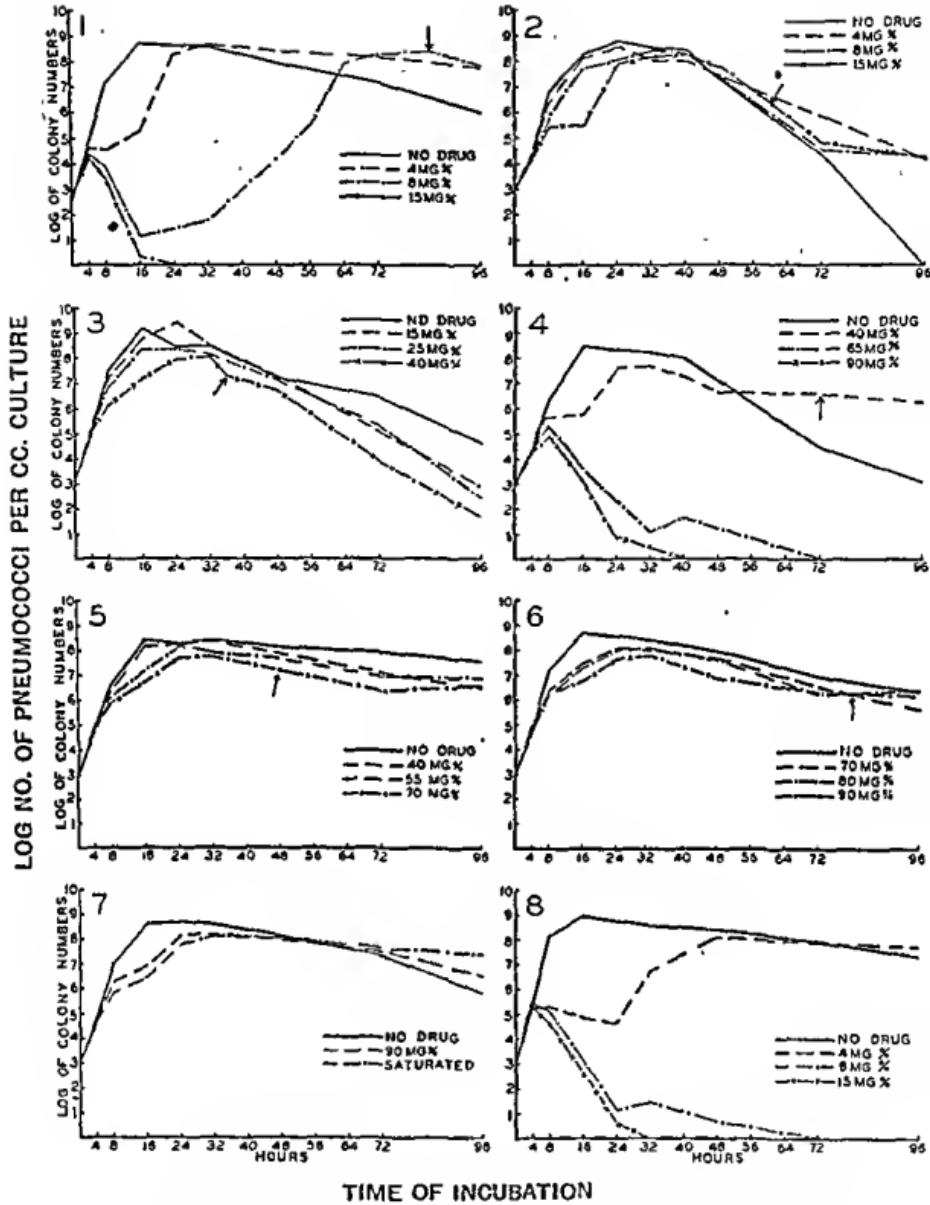


FIG. 2. DEVELOPMENT OF SULFAPYRIDINE RESISTANCE IN TYPE II PNEUMOCOCCUS, STRAIN CH

CHART 7. Effect of sulfapyridine on growth of organisms isolated from brain tissue parent strain after seven serial passages

be decreased slightly following such treatment, since the passaged strain failed to grow in media containing 8 mgm. per cent sulfapyridine, whereas the original parent organism had been able to multiply.

*C. Comparison of the in vivo response of the parent and resistant organism.* It seemed of interest to know whether the organisms isolated from the media saturated with sulfapyridine (Chart 7) were resistant to this drug *in vivo*. Before

TABLE 4  
Retention of resistance by type II pneumococcus, strain CH

EX- PERI- MENT	ORGANISMS		NUM- BER OF MICE IN- FECTED	TREAT- MENT	NUMBER OF DEATHS							SUR- VIVAL OF MICE THAT DIED	THIRTY-DAY SURVIVORS				
	Source	Num- ber in infect- ing dose			Days after infection:								Num- ber	Per cent			
					1	2	3	4	5	6	7- 10						
A	Parent organism after 130 mouse passages	250	30 10	SP20* None	0	0	0	0	0	0	19	184	11	37			
					0	10	0	0	0	0	0	30	0	0			
B†	From <i>in vitro</i> ex- per. 7 after 12 mouse passages	1510	30 10	SP20 None	0	29	1	0	0	0	0	38	0	0			
					1	9	0	0	0	0	0	27	0	0			
C‡	From <i>in vitro</i> ex- per. 7 after 90 mouse passages	200	30 10	SP20 None	0	16	14	0	0	0	0	48	0	0			
					0	10	0	0	0	0	0	38	0	0			
D	From <i>in vitro</i> ex- per. 7 after 169 mouse passages	300	20 10	SP20 None	0	20	0	0	0	0	0	41	0	0			
					2	8	0	0	0	0	0	29	0	0			
E	From <i>in vitro</i> ex- per. 7 after 223 mouse passages	280	30 10	SP20 None	0	30	0	0	0	0	0	38	0	0			
					1	8	1	0	0	0	0	31	0	0			

\* SP20 = 20 mgm. sulfapyridine 2, 8, 14, and 22 hours after infection and every 8 hours thereafter for 5 days, or as long as the animals survive.

† Organisms used in this experiment were passaged through untreated mice and cultured in media containing 160 mgm. per cent sulfapyridine for all 12 passages.

‡ Organisms used in this and the subsequent experiments were passaged through untreated mice and cultured in media containing *no sulfapyridine*.

the *in vivo* test could be carried out, however, the mouse virulence of this resistant strain had to be restored. This was accomplished by 12 passages of the organism through normal untreated mice, the blood of these passage mice being cultured each time in media saturated with sulfapyridine. The *in vivo* response of this organism to sulfapyridine was then studied, the infection and treatment procedure being identical with that used in the *in vivo* experiments described earlier in this report. A similar experiment with the parent type II strain was carried out simultaneously.

The results of this study (Experiments A and B, table 4) show that the organism that had been made sulfapyridine-resistant *in vitro* was highly resistant *in vivo*.<sup>2</sup> The comparison between the responses of the resistant and parent strains is striking. None of the mice infected with the resistant organism recovered and the average survival time of such mice was only 38 hours. On the

TABLE 5  
Retention of resistance by type III pneumococcus, strain CHA

EX- PERI- MENT	ORGANISMS		TREAT- MENT	NUMBER OF DEATHS							SUR- VIVAL OF MICE THAT DIED	THIRTY-DAY SURVIVORS			
	Source	Num- ber in in- fecting dose		Days after infection:								Num- ber	Per cent		
				1	2	3	4	5	6	7- 10					
A	From exper. F (table 1) after 26 mouse pas- sages	400	30	SP20*	0	10	9	2	0	0	0	47	0	0	
			10	Noae	8	2	0	0	0	0	0	23	0	0	
B	From exper. F (table 1) after 44 mouse pas- sages	480	30	SP20	0	14	15	1	0	0	0	48	0	0	
			10	Noae	6	4	0	0	0	0	0	26	0	0	
C	From exper. F (table 1) after 73 mouse pas- sages	750	20	SP20	0	11	8	1	0	0	0	47	0	0	
			10	None	6	4	0	0	0	0	0	24	0	0	
D	From exper. F (table 1) after 142 mouse pas- sages	740	25	SP20	0	10	14	1	0	0	0	51	0	0	
			10	Noae	5	5	0	0	0	0	0	25	0	0	
E	From exper. F (table 1) after 215 mouse pas- sages	1200	30	SP20	1	19	10	0	0	0	0	45	0	0	
			10	None	8	2	0	0	0	0	0	24	0	0	

\* SP20 = 20 mgm. sulfapyridine 2, 8, 14, and 22 hours after infection and every 8 hours thereafter for 5 days, or as long as the animals survive.

other hand, 11 of the mice infected with the parent organism recovered, and the average survival time of the remainder was 184 hours. It should be noted that

<sup>2</sup> It should be pointed out that pneumococci that have been made sulfapyridine-resistant *in vitro* are not always resistant *in vivo*. Thus Seeler and Schmidt (12) have found that 3 strains of pneumococci, which had been made highly resistant to sulfapyridine, sulfathiazole and sulfanilamide by an *in vitro* technique somewhat different than that described above, were only slightly more resistant to these drugs *in vivo* than were the parent organisms.

these strains were about equally invasive, the death times of untreated mice being essentially the same.

**RETENTION OF RESISTANCE.** It seemed of considerable interest to know how long the above strains retained their resistance to sulfapyridine. Accordingly, the various resistant strains were passed repeatedly through untreated mice, the blood of these animals being cultured at each passage in infusion broth containing no drug. At various intervals the *in vivo* responses of these organisms were tested by means of the procedures outlined in the previous experiments.

All 4 resistant strains, type I McGovern, type II CH, and type III, Wistuba and CHA, reacted in a similar manner; consequently only the results with the type II strain made resistant *in vitro*, and with the type III CHA strain made resistant *in vivo*, are presented here. As the data in tables 4 and 5 show, passage of type II, strain CH, through 223 normal mice and type III, strain CHA, through 215 mice did not alter the resistance of these strains to sulfapyridine. Thus it must be concluded that sulfonamide resistance is a characteristic which is retained almost indefinitely once it is well established. However, as a previous study has shown (5), resistance can be lost when this characteristic is only partially developed.

**Discussion.** As has been mentioned previously, our earlier experiments (1, 2) showed that under certain conditions sulfanilamide and sulfapyridine inhibit growth of pneumococci for a limited time only. Other investigators, working with pneumococci (10, 13), streptococci (14) and Brucella (15), have observed this same phenomenon in varying degree. Green (15) attributed the late outgrowth of Brucella to an increase in the amount of a growth-stimulating substance termed "P" factor. The data in the present experiments show clearly, however, that the late outgrowth of pneumococci is due primarily to the development of sulfonamide-resistant organisms, rather than to a change in the composition of the medium.

One of the first questions raised by the present study concerns the mechanism involved in converting sulfonamide-sensitive strains of pneumococcus into resistant strains. The current experiments have shown that the development of resistance is a progressive process, and that when resistance is well established it is retained by innumerable succeeding generations of organisms. Dubos (16) has pointed to these phenomena as characteristics of a process of selection. It seems probable, therefore, that the resistant strains are developed by selective propagation of sulfonamide-resistant variants.

How these resistant variants are formed originally is a question that cannot be answered with the data available. However, judging from experiments on the formation of yeast variants (17), one may suggest that sulfonamide-resistant variants may be formed either during normal multiplication of the sensitive parent organisms, or in response to some injurious action of the sulfonamide. There is a certain amount of evidence that can be interpreted as supporting each of these possibilities. Thus, Frisch (18) observed that pneumococci obtained from a single sputum sample of an untreated pneumonia patient exhibit varying degrees of resistance to the sulfonamides. This observation may indicate that

organisms possessing varying degrees of resistance are formed during normal multiplication of pneumococci. On the other hand, MeKinney and Mellon (19) found that pneumococci exposed to sulfonamides do undergo dissociative changes, and Beall (20) reported recently that exposure to sulfanilamide alters the chromosomal arrangement of certain plants; these observations suggest that sulfonamides may induce mutations, and it is possible that a sulfonamide-resistant organism may be one of the mutants formed. It should be pointed out, however, that the process of selection following appearance of the first resistant variants would probably be the same, regardless of the manner in which the variants are formed.

It may be questioned whether the resistant variants formed directly from the sensitive parent organisms are fully resistant to sulfapyridine. The results of the experiment with type II, strain CH (fig. 2), suggest that these first variants are only mildly resistant. When these mildly resistant variants are exposed to concentrations of sulfapyridine that inhibit their growth, they give rise to more highly resistant organisms, just as did the parent pneumococci. By repeating this process it is possible to eliminate sulfonamide-sensitive organisms entirely and to obtain a culture made up exclusively, or almost exclusively, of organisms that can multiply rapidly in the highest concentrations of sulfapyridine that can be maintained in the body fluids of animals or in artificial media.

It is noteworthy that the superficial properties of the resistant and sensitive organisms studied in this laboratory seem to be identical. These properties include morphology, capsule formation, type specificity, inulin fermentation, bile solubility, growth on blood agar, growth rates in beef heart infusion broth, and virulence and invasiveness for mice.

The above observations raise the question as to what characteristics of the resistant and sensitive organisms determine their responses to sulfonamides. This question cannot be answered satisfactorily at present. It may be suggested, however, that sulfonamide resistance might well be related to any one of the following properties: (1) *Capacity of the organisms to inactivate the sulfonamides.* Inactivation of sulfonamide might be due either to the conversion of the drug into an inactive derivative (perhaps similar to the acetyl form) or to the production of sulfonamide-inhibiting substances like *p*-amino-benzoic acid. There is little information on the production of inactive sulfonamide derivatives by bacteria. On the other hand, sulfonamide-inhibiting substances have been found in a variety of bacteria and yeasts (15, 21, 22); and it is pertinent that MacLeod (23) found more sulfonamide inhibitor in the culture supernatant of a resistant type I pneumococcus than in similar material from a sensitive type I strain. (2) *Capacity of the organism to convert the natural sulfonamide into its hypothetical "active" form.* Several investigators (24-27) have suggested that the active principles of the sulfonamides are the oxidation products rather than the "natural" forms. This has not been demonstrated conclusively, but if it were true, then those organisms that were unable to bring about this oxidation would be sulfonamide-resistant. (3) *Growth requirements of the organism.* Assuming that the sulfonamides inhibit growth by preventing utilization of an

essential growth material in the culture medium (22), then organisms that are sulfonamide-resistant must be able either to synthesize this growth essential, or to utilize some substitute material whose utilization is not blocked by the sulfonamides. (4) *The intermediary metabolism of the organism.* If sulfonamides inhibit growth by blocking an essential metabolic process, then the resistant organism must have a different mechanism for carrying out this reaction than the sensitive organism. MacLeod's observations (28) have suggested that there are differences in the intermediary metabolism of resistant and sensitive strains. According to this investigator, both strains dehydrogenate glucose at equal rates, but the sensitive organisms dehydrogenate lactate, glycerol and pyruvate, whereas the resistant strains do not do this. In this connection it is noteworthy that sulfapyridine interfered with the dehydrogenation of the 3 carbon atom compounds but not with that of glucose.

Critical studies of these various possibilities are now in progress. It may be pointed out that an explanation for sulfonamide resistance might assist materially in understanding the mode of action of sulfonamide drugs.

In conclusion, it should be pointed out that the finding that pneumococci can acquire resistance to sulfapyridine has practical as well as theoretical implications. Studies in pneumococcal pneumonia (8, 29) and pneumococcal meningitis (30) have indicated that the resistance of pneumococci to sulfonamides does increase sometimes during the clinical use of these drugs. Although this problem has not been serious as yet, it seems likely to assume greater importance when these resistant organisms become disseminated by their carriers<sup>3</sup>. This may curtail the value of sulfonamide therapy seriously--especially since pneumococci that have become resistant to one sulfonamide, such as sulfapyridine, are resistant also to other sulfonamides such as sulfathiazole, sulfamethylthiazole, and sulfadiazine (8, 31).

#### SUMMARY

(1) One strain of type I and two strains of type III pneumococcus were made highly resistant to sulfapyridine by serial passage through mice treated with less than curative doses of this drug. The organisms that were made resistant *in vivo* were also resistant to sulfapyridine *in vitro*.

(2) A sulfapyridine-sensitive strain of type II pneumococcus was made highly resistant to this drug by serial passage through broth containing increasing concentrations of sulfapyridine. This resistant organism was also insensitive *in vivo*.

(3) Sulfapyridine resistance was retained by the above strains for more than 200 passages through untreated mice.

(4) The manner in which resistant strains are developed and possible explanations for sulfonamide resistance were mentioned and discussed.

<sup>3</sup> Sulfonamide-resistant pneumococci have been isolated from nose and throat cultures of patients as long as four months after clinical cure of pneumococcal pneumonia (29).

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# THE ISOLATION OF A PROTEIN FROM THE PARS NEURALIS OF THE OX PITUITARY WITH CONSTANT OXYTOCIC, PRESSOR AND DIURESIS-INHIBITING ACTIVITIES<sup>1</sup>

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Following the discovery of the pressor, oxytocic, and renal effects of posterior pituitary extracts, there has been considerable controversy with regard to the number of substances responsible for these effects. Abel and his collaborators (1, 2) maintained that the actual hormone is a "unitary" substance containing all the activities whereas Dudley (3) early showed that at least partial separation of oxytocic and vasopressor activities could be effected. After Kamm and his colleagues (4) had achieved practically complete separation of these two activities, there was general acceptance of the view that at least two active principles (oxytocic and vasopressor) occur separately in the *pars neuralis*. This view was further strengthened by confirmatory experiments by other methods such as those devised in Stehle's laboratory (5, 6). However, Abel (2) did not withdraw his contention that the true hormone is one substance but did admit that two active and separable principles could be extracted from the gland by appropriately drastic procedures. Rosenfeld's (7) ultracentrifugation of chilled and clarified press-juice of ox posterior lobes also supported the view that oxytocic and vasopressor activities are linked together in a single fairly large molecule and may be liberated from the more complex unit by heating at about pH 4.0.

The experiments reported here are believed to demonstrate that a pure protein of the *pars neuralis* can be isolated containing constant amounts of oxytocic, pressor, and diuresis-inhibiting activities in ratios resembling those found in simple extracts of the gland. In the discussion, possible objections to this belief will be considered with regard to both the physico-chemical (e.g. the possible rôle of adsorption) and the physiological aspects of the problem.

**METHODS.** *Physico-chemical methods.* All determinations of pH were made by means of a glass electrode and were accurate to  $\pm 0.02$  pH. The determinations of nitrogen, except those of table 2 for which a micro-Dumas method was employed, were all made by a micro-Kjeldahl method by which as little as 100 micrograms of N could be determined with an accuracy of  $\pm 2$  micrograms. In addition to micro-determination of total sulfur, sulfur

<sup>1</sup> A preliminary report was published in the Proceedings of the American Physiological Society (Amer. J. Physiol., 133: 473, 1941).

distribution (cysteine, cystine, methionine, and sulfate) was investigated by the Kassel and Brand modification of the Baernstein method (8). Anson's method (9) was used to detect sulphydryl groups (cysteine). Solubility determinations were made at room temperature. The electrophoretic characteristics of the protein were determined in the apparatus of Tiselius (10); the electrophoretic patterns were photographed by the method of Longsworth (11). Ultracentrifugation of solutions of the protein was performed in a Bauer and Pickels type (12) of ultracentrifuge driven by an air turbine of the turreted type. The rate of sedimentation was calculated from "schlieren" patterns photographed by Philpot's method as modified by Svensson (13). In several experiments ultracentrifugation of solutions of the protein in separation cells was performed.

*Biological methods.* Estimates of biological activity were all made in terms of U.S.P. reference standard of posterior pituitary powder. Oxytocic activity of various fractions was determined by two methods: the response of the isolated guinea pig uterus or the depressor effect on the fowl's blood pressure. The "physiological" solution used for the isolated uterus was that of van Dyke and Hastings modified to contain 0.75 mM of Ca per liter (14). Furthermore no Mg was added to the fluid since this cation has been found to increase the uterine response to the vasopressor principle (15). In determining oxytocic action by the depressor effect of extracts on the fowl's blood pressure, the method of Coon (16) was largely followed. Dogs and less frequently cats were employed to estimate the vasopressor activity of extracts. Doses were kept small and repeated at sufficiently infrequent intervals to avoid tachyphylaxis. All determinations of blood pressure in the fowl, dog, and cat were made with the glass capsule manometer of Anderson (17). Clotting in cannulas was prevented by the use of a compact modification of Trendelenburg's apparatus (18) to infuse slowly isotonic saline containing 15 mgm. per cent of heparin. To anesthetize cats or chickens 200 mgm. of phenobarbital sodium per kilogram body weight were injected intraperitoneally (cat) or intramuscularly (fowl); dogs were anesthetized by the intraperitoneal injection of 1 ml. per kilogram of 40 per cent alcoholic solution of chlorbutanol as recommended by Kamm and his co-workers (4).

In estimating the inhibition of diuresis in rats we modified Burn's method (19) only to the extent of administering fluid (0.3 per cent NaCl) intraperitoneally instead of by mouth. As a rule either 28 or 32 rats (7 or 8 groups) were used at one time. Half of the groups received the standard and half received the extract to be assayed; several days later the same doses of the same standard or extract were administered in reverse order. Therefore, comparison of the extract and standard was made in the same groups of rats (7 or 8 groups of 4 rats each) as nearly simultaneously as possible.

The melanosome-dispersing action of extracts was compared with U.S.P. reference standard in frogs. For this purpose, normal frogs or frogs hypophysectomized by the method of Teague, Noojin, and Geiling (20) were used.

**THE PREPARATION OF THE PURE PROTEIN FROM EXTRACT OF POSTERIOR LOBES OF OXEN.** A diagram of the method of preparation is reproduced in figure 1. The initial suspension of freshly dissected posterior lobes obtained from frozen pituitaries contains 1 kgm. of tissue in 9 l. of cold 0.01 N H<sub>2</sub>SO<sub>4</sub>. The pH of the suspending liquid varies little (about  $\pm 0.1$  pH) from the value given if different batches are compared. After the mixture has been thoroughly stirred by an electric motor it is allowed to stand in a refrigerator (4°C.) overnight. The separation of the residue and of all subsequent precipitates from supernatants is accomplished by centrifugation. Precipitation of native protein by the addition of 80 grams NaCl to each liter of supernatant adjusted to pH 3.90 (step 2) is also allowed to continue overnight at 4°C.

The last step by which complete purification is achieved is repeated until

solubility is constant. This step is carried out at room temperature which probably is an important variable (20 to 25°C.) affecting solubility. In this final step, constant solubility rather than a solubility of exactly 100 micrograms of N per ml. is sought. In our experience the apparently pure protein may have a solubility as low as 80 micrograms of N per ml.; however, this solubility is constant in a solvent made as exactly as possible like that described in figure 1 in the step next to the last.

The method of extraction is not of high efficiency in terms of the total activity available (about 200,000 units per kg. fresh posterior lobes). The residue

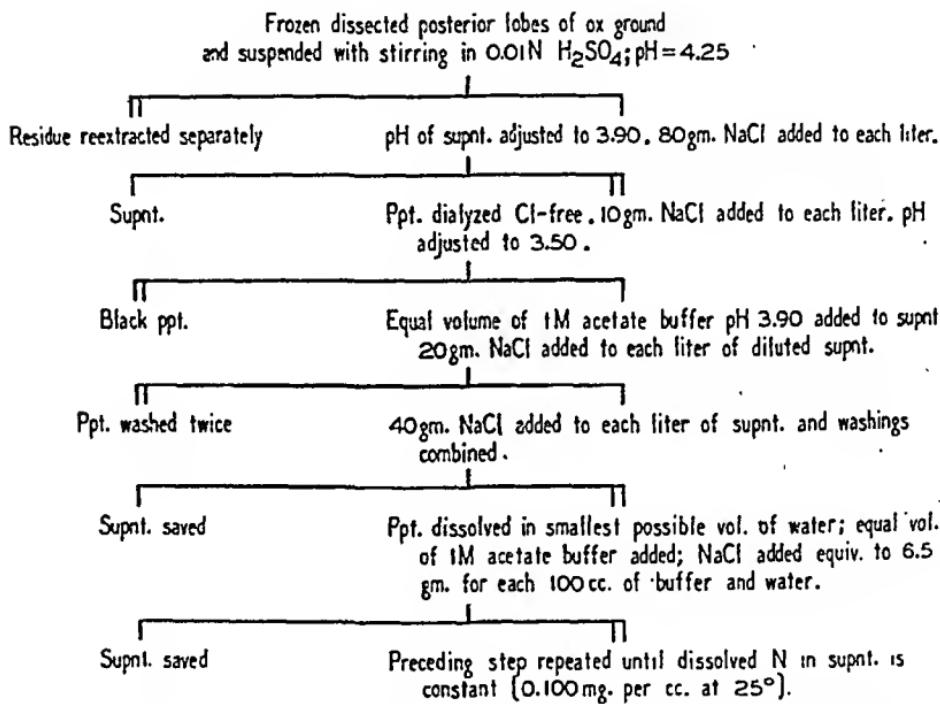


FIG. 1. METHOD OF PREPARING A PURE PROTEIN WITH MULTIPLE ACTIVITIES BY EXTRACTION OF FRESH POSTERIOR PITUITARIES OF OXEN

after initial extraction contains 20 to 25 per cent of total activity which can be removed by boiling a sample of the ground dissected lobes in 0.25 per cent acetic acid. About two-thirds of the 75 to 80 per cent total activity in the supernatant liquid separated from the residue is precipitated by the addition of 80 grams of NaCl to each liter. Therefore, about 50 per cent of total activity is present in the precipitate at the end of the second step. During dialysis of this precipitate activity which apparently is non-protein is also lost; however, we have not attempted to estimate this loss accurately. From 1 kgm. of fresh glands about 700 mgm. of pure protein (>11,000 units) can be isolated apart from subsequent recoveries in supernatants of the last two steps. Samples of the pure protein so far isolated are amorphous.

EVIDENCE THAT THE PROTEIN ISOLATED IS PURE. *Constant solubility.* Northrop and his collaborators (21) in studying crystalline enzymes have applied with great success the solubility test of Sørensen in determining the presence of small amounts of impurity or in demonstrating homogeneity of crystalline enzymes. The solubility characteristics of the protein isolated in the example of figure 1 are shown in figure 2. The solvent used was 0.5 M acetate buffer, pH 3.90, to which 6.5 grams of NaCl were added to each 100 ml. It is clear that by this test there is no evidence that more than one component is present either before the solvent is saturated with the protein or after twenty times the saturating concentration is in suspension. The amounts of protein which have been available have not permitted us to carry out solubility tests in other solvents which should be employed. We have already mentioned that at different times or with other preparations, constant solubility in the same solvent made later might be as low as 0.080 mgm. of N dissolved in each ml. Variables such as

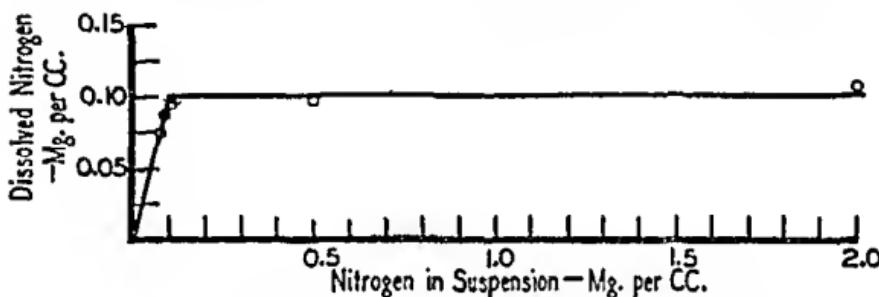


FIG. 2. SOLUBILITY OF THE PURE PROTEIN IN 0.5 M ACETATE BUFFER, pH = 3.90, TO WHICH 6.5 GRAMS NaCl WERE ADDED TO EACH 100 ML.

room temperature or slight changes in the pH of the buffer (e.g., pH 3.95 instead of 3.90) probably explain the variation in absolute amount of protein dissolved.

*Electrophoretic homogeneity.* A large number of electrophoretic patterns of the pure protein have been photographed in the Tiselius electrophoresis apparatus. In figure 3, mobility per centimeter per second per volt per centimeter has been plotted against pH. The isoelectric point of the protein appears to be about pH 4.8. Examples of patterns photographed by the method of Longsworth (11) are shown in figure 4. The upper three patterns were made with three different preparations of pure protein at pH 3.41-3.47. At this pH only, of those used (fig. 3), there appears to be a second protein which is indeed small in comparison with the main component. The oxytocic activity of this minor substance is no greater than that of the main component in terms of nitrogen. At other pHs (e.g., preparation XI-85-G at pH 6.05) there is electrical inhomogeneity but no second protein can be separated. It appears that the minor component at pH 3.4-3.5 is closely related to the main component and possibly is derived from it.

*Studies of the protein in the ultracentrifuge.* In the ultracentrifuge this material appears to be a single homogeneous protein with a molecular weight of about

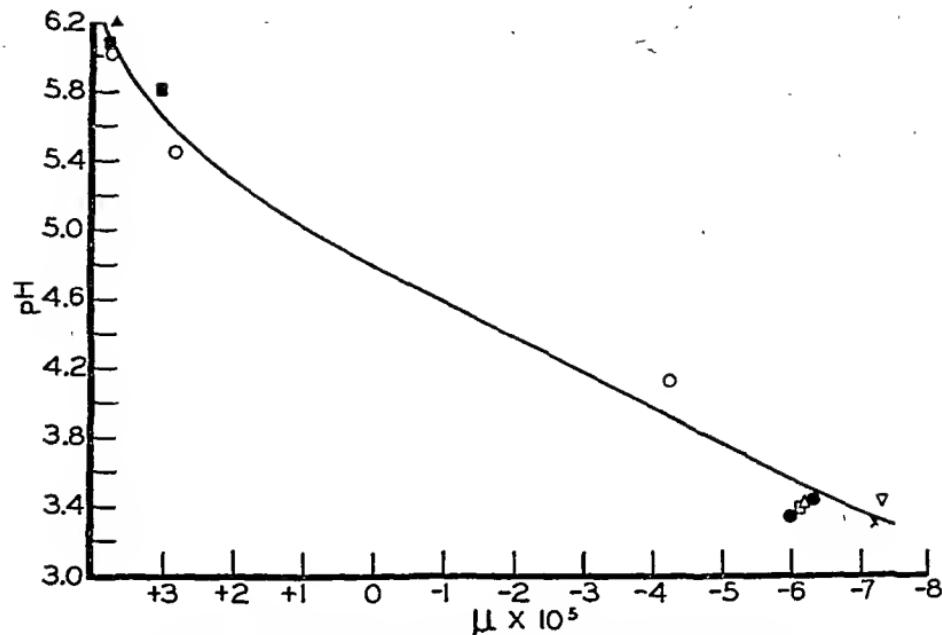


FIG. 3. MOBILITY OF THE PROTEIN IN RELATION TO pH AS DETERMINED IN THE ELECTROPHORESIS APPARATUS OF TISELIUS

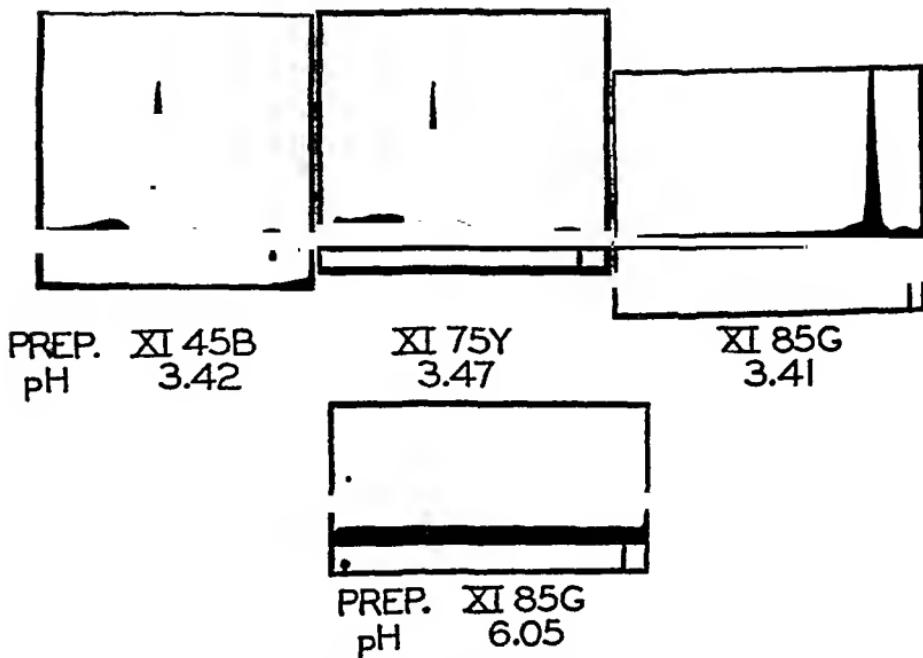


FIG. 4. ELECTROPHORETIC PATTERNS OF THE PROTEIN. SEE TEXT

# A METHOD FOR THE BIO-ASSAY OF DIGITALIS IN HUMANS

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Evidence has accumulated in recent years which indicates that animal methods for the assay of digitalis and its glycosides give results which may be misleading when applied to humans (1). It has been shown that the frog method has serious defects in this respect, since a specimen which has the same activity as another when compared in humans, may be only half as strong as the other when compared in frogs. In these studies the results of the cat method proved to be a more reliable index of the relative potency of digitalis preparations in man, but defects of the cat method were also pointed out. In the past two years we have explored the possibilities of using human subjects for the assay of digitalis. The present report deals with a description of a satisfactory human method of assay which has been developed in the course of these studies.

Attempts have been made in the past to compare digitalis preparations in humans directly, but these have been for the most part abortive. Pardee (2) suggested the use of the *T*-wave of the electrocardiogram as a measure of digitalis action in humans, and the minimal dose necessary to produce a distinct lowering of the *T*-wave as a means of comparing a stronger with a weaker preparation. In this way he compared four tinctures of digitalis and obtained some indication that one specimen was weaker than the others, although the method was not developed in such a manner as to reveal the extent of the difference. The comparisons of two specimens were made on different groups of patients. Martin (3) attempted to compare different specimens of digitalis in man by means of the dose necessary to produce therapeutic effects in patients with heart failure. These patients received a fairly large dose on the first day and additional doses on subsequent days until a therapeutic effect was in evidence. The average amount of one specimen required for one group of patients was compared with the average amount of another specimen required in another group of patients. In this way he compared specimens A, B, and C, which were supplied by the Hygiene Committee of the League of Nations. The average doses for the three specimens were different, suggesting that their potency was different. In the light of the marked individual differences within the group and the fact that the end point (therapeutic effects) is not a very sharp one, it is open to question whether the differences in the doses are significant as an index of differences in the potency of the preparations. Gilchrist and Lyon (4) also made a comparison of the foregoing three specimens of digitalis in a larger group of patients. They used as the criterion the amount of slowing of the heart in patients with auricular fibrillation, each patient receiving the same amount of drug in relation to his

body weight. The results of this study also suggested that specimens A, B, and C were of different potencies, but the ratios were different from those obtained by Martin with the same specimens and a different method. Again, the comparisons of the three specimens were made in different groups of patients. In none of these studies was the sensitiveness of the method tested.

An interesting attempt to calibrate the subject was made by Dieuaide, Tung and Biou (5). They used one preparation and gave three doses at intervals of three weeks or longer to each of several patients. These observers seemed to be able to distinguish a 25% difference in dosage by the degree of shortening of the Q-T interval of the electrocardiogram. One of their subjects showed a striking three point calibration; with the smallest dose the change in the value of  $K^1$  was 0.01; with the middle dose, 0.05; with the largest dose, 0.08. The method presents interesting possibilities for the assay of digitalis on humans, and we propose to explore this end-point in the electrocardiograms of our study.

The method we have used is based on the well-known fact that the degree of *T*-wave and *RT-T* changes in the electrocardiogram increases with the dose of digitalis. It should be noted that in a recent report, Geiger, Blaney and Druckemiller (6) stated that electrocardiographic changes cannot be correlated quantitatively with the amount of the drug. A detailed analysis of their paper would not prove profitable at this point. Their method of study was sufficiently different from ours to account for the contrary conclusion. DeGraff (7) has expressed the opinion that the electrocardiogram cannot be used for the assay of digitalis in man for a reason that has no bearing on the method we employ. In a previous study (8) we showed that a comparison of two digitalis materials in man by means of the electrocardiogram yields the same result as when the comparison is made by means of one of the best-known therapeutic effects, namely slowing of the ventricular rate in auricular fibrillation. Furthermore, evidence will be presented in the present paper showing that the electrocardiogram may be used to establish a satisfactory dosage-response curve in properly selected subjects.

**METHOD.** The patients used in this study were selected from a case load of about 1500 patients in attendance in our cardiac clinics. They were all ambulant. Most of them had organic heart disease, and all the common varieties were included, rheumatic, arteriosclerotic and hypertensive heart disease. They all had regular sinus rhythm. None had heart failure. A wide range of ages, from 17 to 65, and both sexes are represented. Most of them had normal electrocardiograms. Those with abnormal ones proved, as a rule, to be unsatisfactory for our purposes because of their tendency to rather marked spontaneous variations in the form of the deflections.

In the present technique the plan was first to calibrate patients with three doses of the U.S.P. Reference Digitalis Powder, differing from each other by 22%. The dose of the unknown preparation was then selected which was calculated to produce an effect falling within the calibrated range, but not coinciding with the two extremes. The ratio of the dose of the unknown to the dose of the standard was taken to represent the relative potency of the two preparations in a single subject. The average of these ratios for a series of six or more patients established the potency of the unknown in relation to the standard.

All electrocardiograms were taken with the patient resting and in the same position. A

<sup>1</sup> *K* expresses the *Q-T* interval in relation to the cycle length.

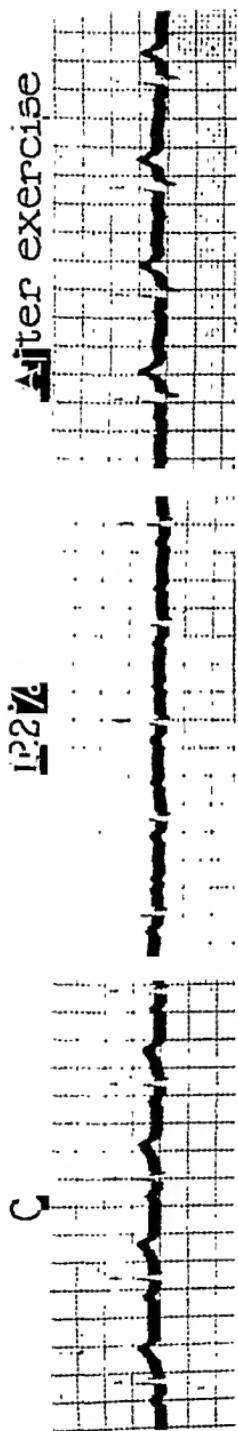


FIG. 1. FACTORS INFLUENCING THE EFFECT OF DIGITALIS ON THE *T*-WAVE  
Patient Fr. Fr. (C) control. (122%) 24 hours after 732 mg. U.S.P. Reference Digitalis Powder; note the striking change in this tracing after exercise. Lead 1.

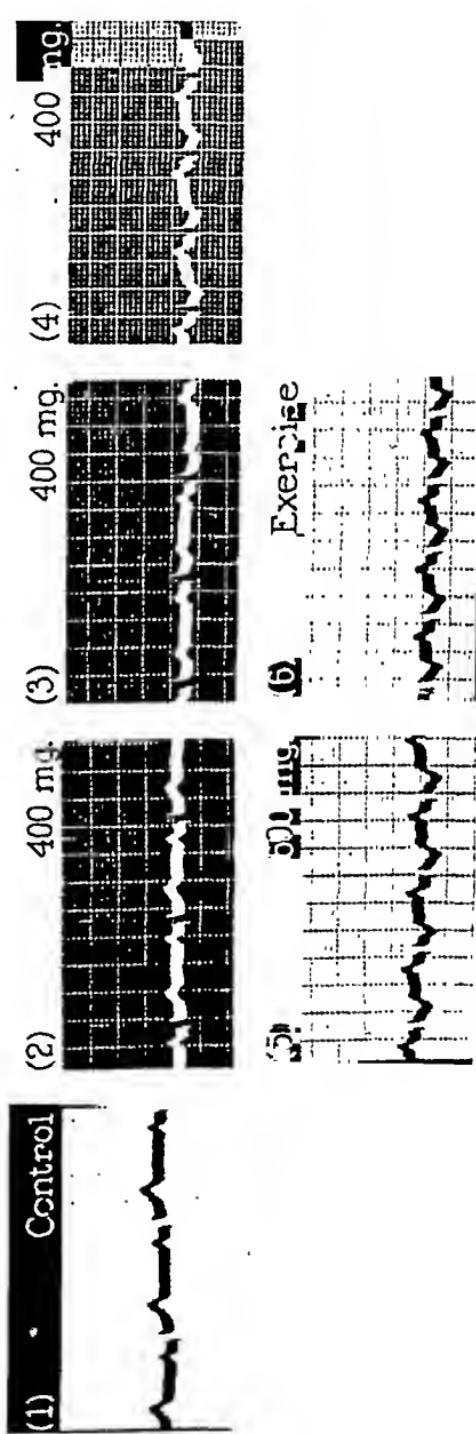


FIG. 2. FACTORS INFLUENCING THE EFFECT OF DIGITALIS ON THE *T*-WAVE  
Patient Mc. Ro. Note the three grades of *T*-wave change produced by the 400 mg. dose of U.S.P. Reference Digitalis Powder (tracings 2, 3, 4). The greatest effect caused by the 400 mg. dose (tracing 4) is similar to the effect of the 600 mg. dose (tracing 5). Tracing 2 was converted into tracing 6 by exercise. Lead 2.

control electrocardiogram was taken, and immediately thereafter a dose of U.S.P. Reference Digitalis Powder was given orally in the form of capsules. The effect of this dose was observed in the electrocardiogram taken 24 hours later. Such an experiment was repeated at intervals of 4 weeks with different doses. The object was to discover the most sensitive range in the dosage-response curve. The test with the unknown preparation of digitalis was carried out in the same manner as with one of the doses of the standard; after a control electrocardiogram, a dose of the unknown was administered and its effect upon the T-wave determined from the electrocardiogram taken 24 hours later.

For reading the electrocardiograms a section of the records made from lead I and lead II was mounted on a 4 by 6 inch filing card with the legend on the back. For each patient

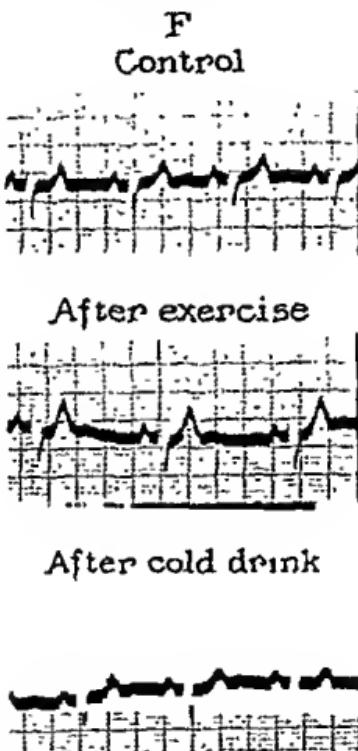


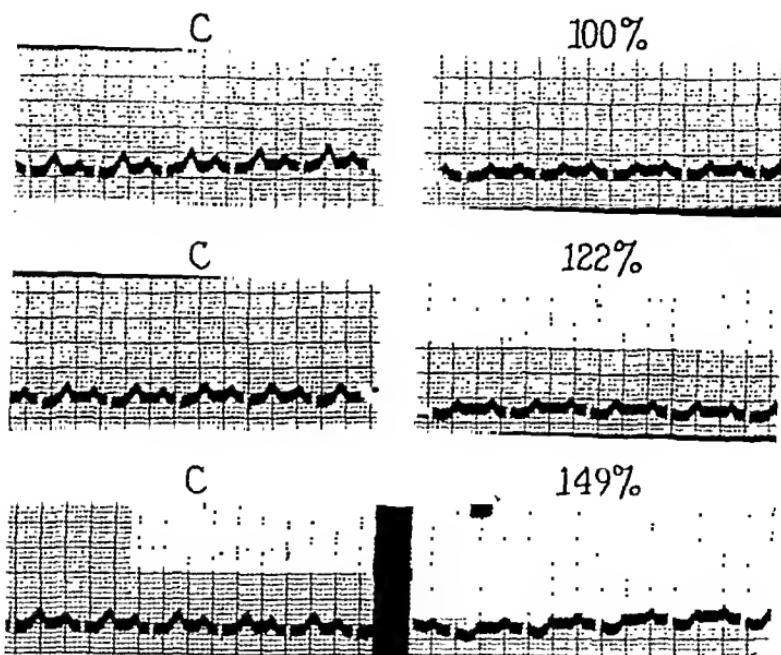
FIG. 3. FACTORS INFLUENCING THE HEIGHT OF THE NORMAL T-WAVE

Patient Lo Fa. Note the marked increase in the amplitude of the T-wave with exercise, and decrease with a drink of cold water. Note also that the effect of exercise on the T-wave persists in the period of secondary slowing. Lead 2.

there were at least 8 such cards, one before and one after each of the three calibration doses, and one before and one after the dose of the unknown preparation. Five of these cards, including one control, were examined by an individual expert in the reading of electrocardiograms who matched and ranked them according to the degree of effect on the RT-T group by the "blind test", without knowledge of the legends. The ranking was sometimes made independently by a second person. The agreement was, with few exceptions, satisfactory. The lead in which the T-wave was most sensitive to digitalis was used, sometimes lead I, other times lead II. In some cases the decisions were based on the changes in both leads I and II. In patients who proved suitable for assay, the order of ranking corresponded to the order of the doses.

The character of the change in the electrocardiogram produced by digitalis varies from patient to patient and with the size of the dose. The change may be either a lowering of the amplitude of the *T*-wave, or a flattening to depression of the *RT* segment, or a combination of the two. While measurement of the change in millimeters was sometimes used, the tendency to distortion in the contour of the entire *RT-T* group as the result of digitalis makes direct measurement as the sole means of estimating grades of change unreliable, although it is possible that some more complex form of measurement might be devised.

There are several pitfalls in this method of assay of digitalis; and several factors require more extensive study. The control *T*-waves, even in the most favorable subjects, showed a fair amount of variation. The question arose whether the effect of the dose should be related to the control taken 24 hours previously or to any one control tracing. Which



4. THREE-POINT CALIBRATION OF PATIENT WITH U.S.P. REFERENCE DIGITALIS POWDER  
Patient Ro. Da. (100%) 600 mg. (122%) 732 mg. (149%) 893 mg. Note the progressive depression of the *R-T* segment which distinguishes the effects of the three doses. Lead I.

form of comparison gives the truer value has not been determined. We have adopted the procedure of relating the effects of the various doses to each other, using only one representative control, in order to avoid the necessity of assigning an absolute value to any one of the changes. We are uncertain as to whether or not this, in the end, will prove the most reliable technique.

The selection of patients and the conditions under which the electrocardiograms were to be taken presented something of a problem. The *T*-waves of the electrocardiogram, both as regards height and form, are well known to be influenced by many factors: position of the patient, exercise, infection, cold drinks, food, respiration, and many others. Figure 1 shows how marked such influences can be. In this case a dose of digitalis produced marked lowering of the *T*-wave. During the period of rest following exercise, the effect of digitalis

on the *T*-wave completely disappeared, and the *T*-wave became even higher than it was in the control. Such a change could be grossly misleading, since the effect of the exercise on rate was no longer in evidence at the time that this reversal of the *T*-wave change was present.

Figure 2 shows the opposite type of effect of exercise. In this case three tests, each with a dose of 400 mg. of U.S.P. Reference Digitalis Powder, produced widely different effects on the *T*-wave. One of these effects was similar to the effect of the 600 mg. dose. It was

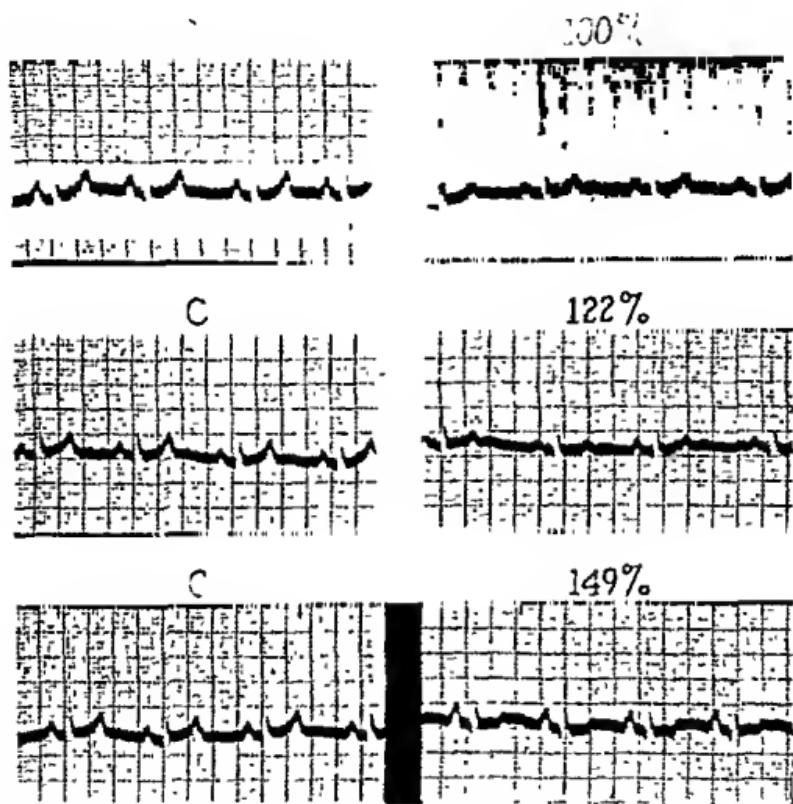


FIG. 5A. THREE-POINT CALIBRATION OF PATIENT SE. GO. WITH U.S.P. REFERENCE DIGITALIS POWDER

Note the distinct grades of change representing 22% differences in dosage. Lead 2. First calibration of Se. Go. in table I.

then found that by means of exercise the effect of the 400 mg. dose could be converted into the deep *T*-wave inversion of the 600 mg. dose.

The numerous factors which influence the electrocardiogram have received a great deal of attention in the literature. We have made several experiments with exercise, with cold drinks, and with changes in the respiration which leave little doubt that these factors, if not taken into account, may vitiate the results of an assay on humans (fig. 3).

There are still other problems. Not all patients with a normal electrocardiogram are suitable subjects for assay. In some the electrocardiogram is insensitive and only negligible changes occur, even with a dose of digitalis which produces vomiting. In some, dif-

ferences in doses of less than 50% can not be detected, and the range of even this degree of sensitiveness is very small, so that a three-point calibration can not be obtained.

In many cases the factors which we have described as influencing the effect of digitalis on the *T*-wave are so readily brought into operation that suitable calibration is virtually impossible. The changes are too irregular in such cases and there is marked variation in the control. Such patients are not suitable for assay.

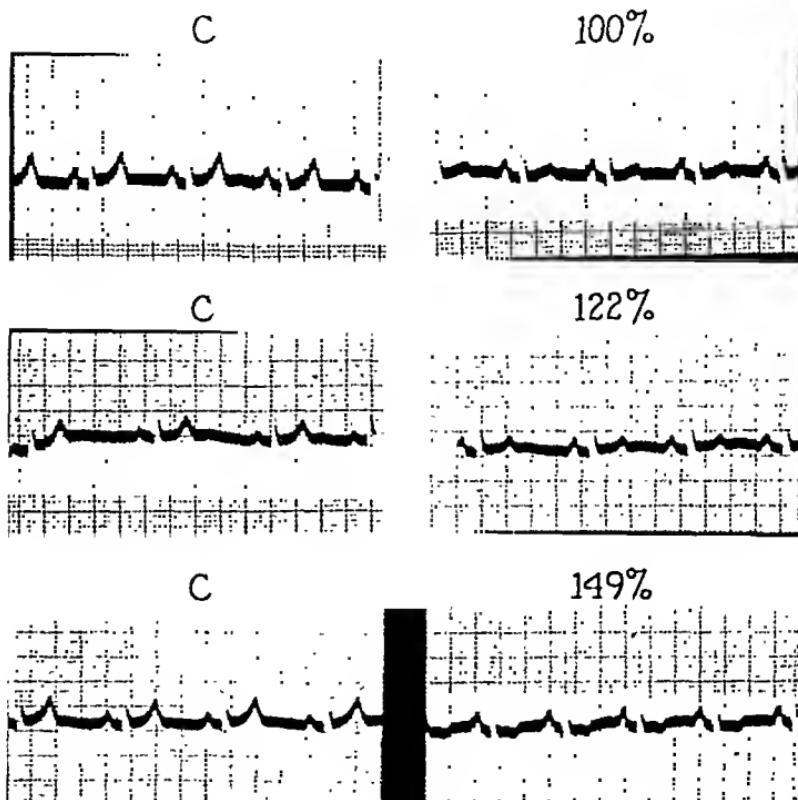


FIG. 5B. THREE-POINT CALIBRATION OF PATIENT SE. GO. WITH U.S.P. REFERENCE DIGITALIS POWDER

This is a second calibration of the patient shown in figure 5A. Lead 2. Second calibration of Se. Go. in table 1.

**RESULTS.** The various factors which we have described greatly reduce the number of available calibrated patients. Thus far our experience involves a total of 97 patients in whom calibration has been attempted. In these, more than 1000 electrocardiograms have been examined. Eighteen of these patients have proved satisfactory subjects for the assay of digitalis.

Figure 4 is an illustration of the best type of subject. This shows a satisfactory three point calibration. The controls in this case are fairly constant. The electrocardiogram is very sensitive to digitalis and shows indisputable changes which distinguish 22% differences in the dose.

Figures 5A and 5B show a similar ease in which the opportunity was afforded for repeating the complete calibration with the standard reference powder in the same patient during a period of 8 months. While the effects are not identical, each calibration gives 3 distinct grades of change in the electrocardiogram to

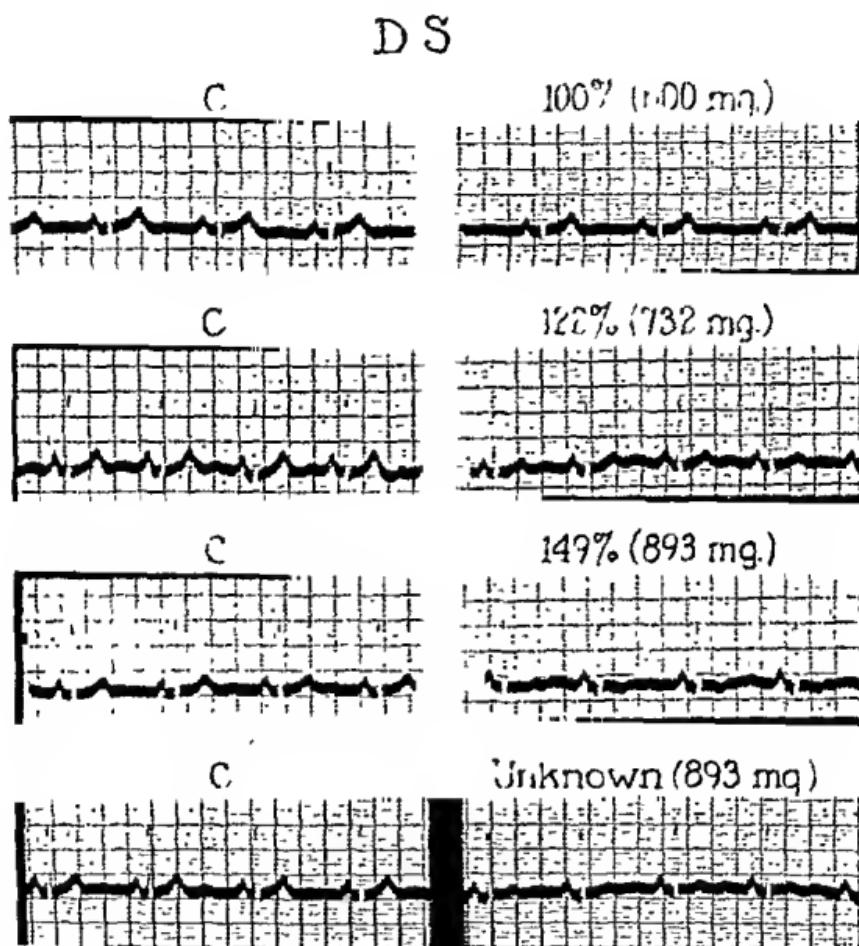


FIG. 6. ILLUSTRATION OF ASSAY OF UNKNOWN SPECIMEN OF DIGITALIS (N) IN A PATIENT CALIBRATED WITH THREE DOSES OF U.S.P. REFERENCE DIGITALIS POWDER  
Patient Da. St. Lead 2

distinguish the 22% differences in dosage. This is in fact a comparison of a preparation of digitalis with itself in one and the same subject.

Figures 6 and 7 are illustrations of the use of calibrated patients in the assay of two unknown specimens of digitalis. In figure 6 the effect of 893 mg. of the unknown approximates the effect of 812 mg. of the U.S.P. Reference Digitalis Powder, since the effect falls somewhere between that of 732 and 893 mg. of the

standard. The unknown specimen has, therefore, a potency of 91% of the standard. In figure 7 the effect of 893 mg. of another unknown is practically

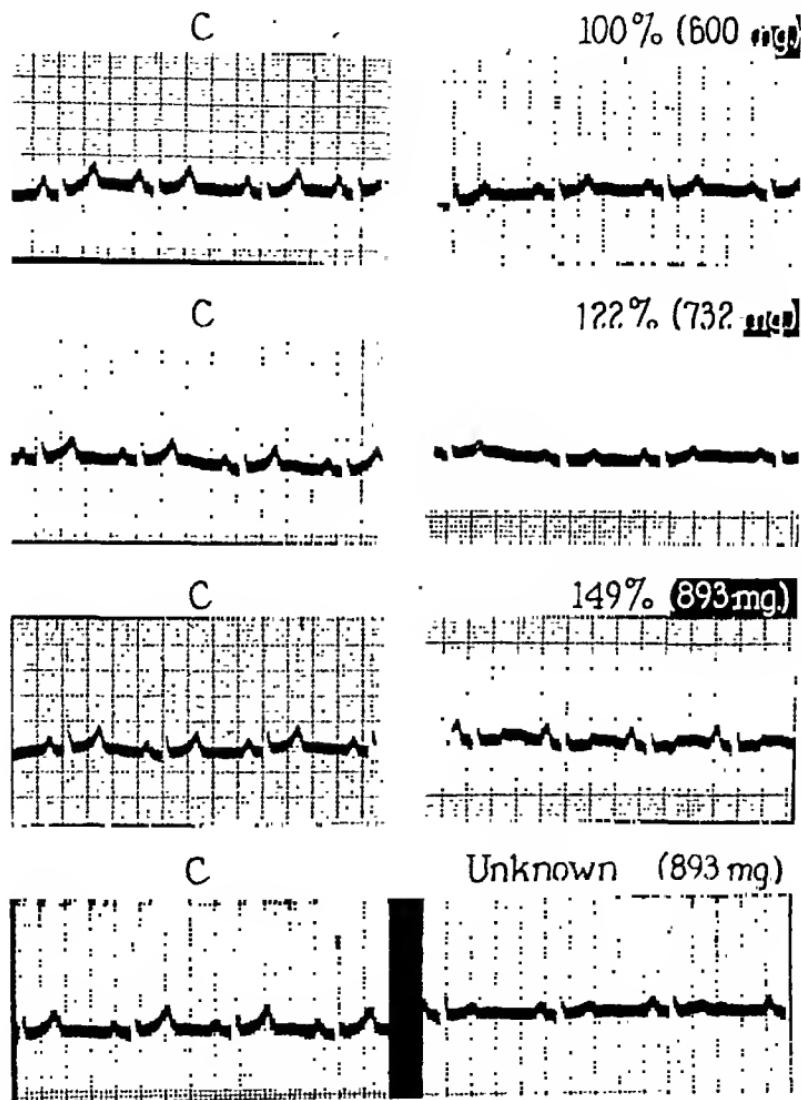


FIG. 7. ILLUSTRATION OF ASSAY OF UNKNOWN SPECIMEN OF DIGITALIS (Y) IN A PATIENT CALIBRATED WITH THREE DOSES OF U.S.P. REFERENCE DIGITALIS POWDER  
Patient Se. Go. Lead 2.

identical with the effect of 732 mg. of the U.S.P. Reference Digitalis Powder. This unknown specimen has, therefore, a potency of 82% of the standard.

Table 1 shows the results of a complete assay of an unknown specimen of digitalis on a series of calibrated patients. It may be noted that 1 mg. of the

unknown powder equals in potency 0.83 mg. of U.S.P. XI Reference Powder, or the unknown is 83% of the potency of the standard.

We are indebted to Dr. W. J. Youden of the Boyce-Thompson Institute for Plant Research, Yonkers, N. Y., for his help in examining the results from the statistical standpoint. Since these data involve the method of ranking and only a few points for each patient, a special problem arises in securing a mathematical expression for the reliability of an assay. The Standard Error is low in the typical assay shown in table 1, namely  $\pm 3.6\%$ , but this expression is less dependable when calculated from an interrupted series of relatively coarse steps. Since the dose of digitalis is increased by steps of 22%, the process of interpolation involves steps of only 11%, and in a series of 6 patients the attainable precision is about 5%. In an experience with 8 specimens of digitalis assayed in the fore-

TABLE 1  
*Human assay of digitalis*

NAME	U.S.P. XI REF. POWDER						UNKNOWN PREPARATION	NO. OF MG. OF UNKNOWN AND DIFFERENCE WHICH PRODUCE SAME EFFECT	DOSE U/DOSE R	POTENCY OF UNKNOWN IN PER CENT OF STANDARD				
	100%		122%		145%									
	mg.	c.	mg.	c.	mg.	c.								
Se. Go.....	1+		2+		3+									
Se. Go.....	600	1+	732	2+	893	3+	893	2+	893 U = 732 R	122	82			
Mc. Ro. . . .	400	1+	488	2+	600	4+	600	3+	600 U = 544 R	110	91			
Da. St... . . .	600	0	732	1+	893	2+	893	1+	893 U = 732 R	122	82			
Ye. Sh... . . .	270	1+	330	1+	400	3+	400	2+	400 U = 365 R	110	91			
Id. Ru. . . .	600	0	732	2+	893	3+	893	1+	893 U = 666 R	134	75			
Ge. Bo... . . .	600	0	732	3+	893	2+	893	1+	893 U = 666 R	134	75			
Average . . . . .										122	83			

\* Effect.

going manner, the ratios for different subjects in any assay usually showed a maximum variation of less than 25%, when the assay fulfilled all the conditions. One specimen of digitalis was assayed 4 times with different groups of calibrated patients, in the form of tablets, capsules, a tincture made from the leaf, and another tincture made at a subsequent time from the same leaf. In percentage of the potency of U.S.P. XI Reference Powder, the results of these assays were strikingly uniform: 82.0, 77.8, 86.9 and 77.5. A degree of reliability to within 25% or better in the human assay is therefore probable.

Up to the present time 8 specimens of digitalis have been assayed in the foregoing manner.

#### SUMMARY AND CONCLUSIONS

1. A method is described for the practical assay of digitalis preparations in humans by means of electrocardiographic changes.

2. The proper selection of subjects is essential. They are ambulant. They should have essentially normal electrocardiograms. The control tracings should be fairly constant. The *T*-waves and *RT* or *ST* segments should be sensitive to 22% difference in dosage.

3. The essentials of the method are the following: A three point calibration of the patient is made with the standard preparation of digitalis. The doses differ by 22%. Each dose is given immediately after a control electrocardiogram. Its effect is determined in the electrocardiogram taken 24 hours later. The effect is a change in the *RT-T* segment of the electrocardiogram. Four weeks elapse between doses. The effects are ranked by the blind test. The unknown is compared with the standard in each of several such calibrated subjects. The potency of the unknown is expressed in terms of the standard and represents the average of the ratios obtained for each of several subjects. If a group of calibrated patients are available, the answer for an assay of an unknown specimen may be obtained within a period of 24 to 48 hours.

4. The many sources of error in this technique are pointed out.

5. This method for the assay of digitalis on humans is now being applied to the standardization of digitalis preparations of commerce.

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# A PRACTICAL TECHNIQUE AND DESIGN FOR THE ASSAY OF DIGITALIS ON THE EMBRYONIC CHICK HEART

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Previous reports (1, 2) have suggested that the embryonic chick heart may be a suitable preparation for the assay of digitalis glycosides and a comparison of lanatoside C, digoxin and digitaline Nativelle by this method gave results which agreed better with the oral human dosage than did those obtained with either the cat or the frog. The object of the present study was, therefore, to standardize a technique which could be carried out on digitalis tincture and whole leaf by an operator without previous training in embryology, to design an assay which would give the maximum information from the biological material used, and to determine the average accuracy and precision which may be expected from the method.

**PROCEDURE** *Incubation.* It is desirable to use eggs from pure bred hens which have all been laid on the same day, and incubation should be started as soon after laying as possible. However, the results have been satisfactory as long as eight days afterwards. Any forced draft incubator with accurate temperature control<sup>1</sup> may be used. In the assays described below the eggs were put into the incubator in lots of 22 every hour for five hours, and the assay was started on each lot 47 hours later. A temperature of  $39^{\circ} \pm 0.5^{\circ}\text{C}$ . was found to bring the embryos to the desired degree of maturity within this time interval, but this value must, of course, be adjusted to meet individual conditions. No humidity control seemed to be necessary for so short an incubation period.

**Preparation of solutions.** Specimens of powdered digitalis whole leaf were made into tinctures by the U.S.P. XI method. In the case of tablets 25 or more were finely powdered and suspended in a volume of the official menstruum which would give roughly one U.S.P. XI digitalis unit per cc. In either case, the samples were shaken continuously for 24 hours, centrifuged and the tincture decanted. All tinctures were preserved at  $5^{\circ}\text{C}$ . in the dark and used within one week of preparation. The U.S.P. XI reference powder was used as the standard and its tincture was in every instance prepared simultaneously with the unknown. Two dilutions each of standard and unknown tinctures were made with Tyrode solution<sup>2</sup> within the range 2.5 and 25 cc. per 10 liters. As will appear below, it is essential that the ratio of high to low concentration for the standard be identical with that for the unknown so that the suggested factorial design will be applicable. A ratio of 2.5 to 3 was usually appropriate. The proper concentrations were determined by a few exploratory experiments and were such that atrioventricular block occurred within 2.8 and 12 minutes. It is desirable to have the hearts block at about the same average time for the low concentrations of standard and unknown, and again for the high concentrations.

**The warm chamber.** An ordinary compound microscope is mounted in the center of a chamber about 12 inches high and with a base about 15 by 15 inches square. This may be

<sup>1</sup> Such as Model 96, American Electric Incubator Company, New Brunswick, N. J.

<sup>2</sup> Percentage composition used in this work was as follows:  $\text{NaCl}$ , 0.8;  $\text{KCl}$ , 0.02;  $\text{CaCl}_2$ , 0.02;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.01;  $\text{NaH}_2\text{PO}_4$ , 0.005;  $\text{NaHCO}_3$ , 0.03 and glucose, 0.1. The pH was 7.4. The solution must, of course, be prepared on the day of the assay.

constructed of wood or any insulating material. It is provided with a hole in the top for the ocular of the microscope and a sliding panel on the right side which allows access to the stage through an opening 5 inches in diameter. A glass window is mounted in the back for illumination. Temperature regulation is accomplished by means of a 60 watt carbon filament lamp for heater, hydrogen sealed mercury thermoregulator, vacuum tube relay to protect the mercury contacts and a 5 inch, 6 volt auto fan with rubber blades to dissipate the heat of the lamp rapidly. Twenty diameters is sufficient magnification and gives a large enough field to allow three hearts to be observed at the same time. Before beginning the dissection a drop of one of the diluted tinctures is transferred to the well of a slide, covered and placed on the stage of the microscope in the warm chamber. The drop has then reached the temperature of the chamber by the time the hearts are ready.

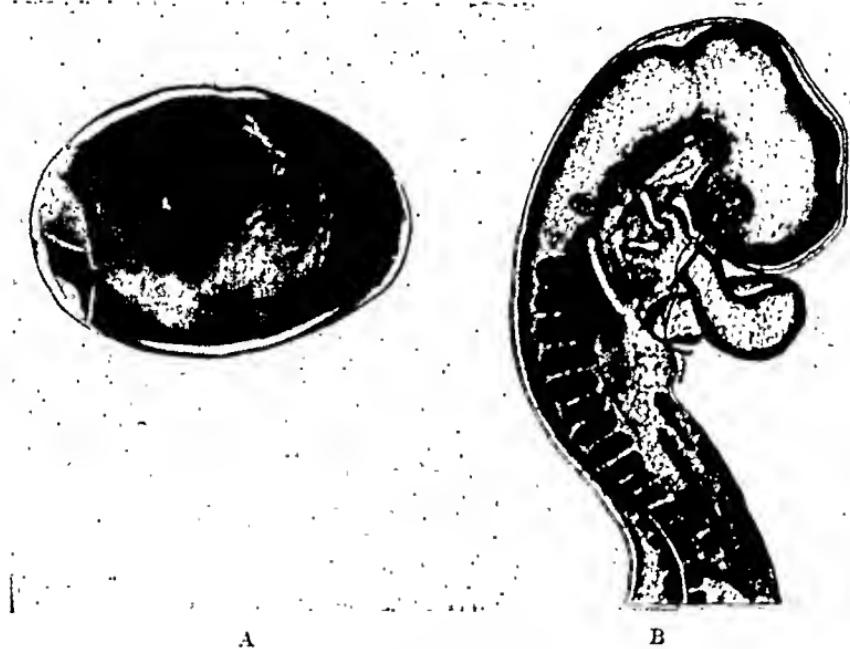


FIG. 1-A. PHOTOGRAPH (5/6 ACTUAL SIZE) OF EGG OPENED TO SHOW EMBRYO LYING ON YOLK

The diameter of the area enclosed by the sinus (between arrows) is used as a preliminary criterion of degree of development. The square indicates the portion which is excised.

B. Drawing of an entire embryo (12 diameters) showing details of the dissection

*Dissection and selection of hearts of desired degree of maturity.* An oval window is cut in the shell of the egg, exposing the yolk. If the embryo is not lying directly in view, as in figure 1A, the yolk may be rotated with the handle of a scalpel. This process is usually aided by pouring off some of the albumen. If the largest diameter of the area included by the venous sinus does not fall within 14 and 18 mm. (between arrows in figure 1A) the egg may be discarded at once as being outside the desired range of maturity. Otherwise, with the embryo in full view a pair of clean moist scissors is used to make four cuts (as shown by the square in figure 1A) in the membranes, one posterior to the heart across the mid-body

\* A well which is approximately 18 mm. in diameter and 1.75 mm. deep, and drops of 0.05 to 0.08 cc. were found to be satisfactory.

region of the embryo, and three more, close enough to the head to avoid inclusion of yolk in the portion to be removed. Avoiding the heart, a pair of moist forceps permits one to lift this region of the embryo off the yolk and place it in a shallow dish of fresh Tyrode solution at room temperature. Two more embryo fragments are cut out in the same way and all three are allowed to wash for a minute or two so as to remove any adherent albumen. They are then transferred to a small lake of Tyrode solution on the stage of a dissecting microscope. In the removal of the hearts two cataract knives are used. With the point of the knife in the region marked with a cross in figure 1B, the amnion is jerked away from the heart. By a scissor-like use of the knives the conus first and then the sino-atrium are transected as indicated respectively by the upper and lower black lines in figure 1B.

The three hearts are now examined for degree of development, in which case figure 2 may be of some help. There are no fool-proof criteria which will substitute for 2 or 3 days of experience gained by actually working with the embryonic heart. In general, however, one can say that in Figure 2A the heart is underdeveloped and is relatively difficult to remove from the embryo because of the presence of the dorsal mesocardium (mesentery). There is little differentiation of an atrium. In figure 2B the dorsal mesocardium



FIG. 2. PHOTOGRAPHS (40 DIAMETERS) TO ILLUSTRATE CRITERIA USED IN RESTRICTING THE HEARTS TO A NARROW RANGE OF DEVELOPMENT

Hearts qualitatively resembling A and C are rejected while those resembling B are used in the assay.

may be considered to be absent, and the heart has reached a degree of development which is considered normal as far as the assay is concerned. The atrium is well formed and the texture of ventricle and conus is quite uniform. In figure 2C the heart is overdeveloped and the texture of ventricle and conus differs because of the appearance of trabeculae in the ventricular wall. Hearts corresponding to 2A and 2C are discarded and replaced by others until three satisfactory ones remain on the stage of the dissecting microscope. The restriction of the hearts according to these qualitative criteria is quite objective and avoids a size measurement which would be prohibitive from a practical point of view. The dissection must be done with dispatch and no more than 6 minutes<sup>4</sup> should elapse between the opening of the first egg and the transfer of the three hearts to the digitalis solution in the well slide. The transfer is accomplished by means of a very thin, flat strainer about 2 mm in diameter. This may easily be fashioned by hammering the end of a fairly heavy gauged platinum wire after which holes are inserted with a steel needle and the edges

<sup>4</sup> In case many eggs have to be discarded because of infertility or improper development it is better to determine the end point with one or two hearts at a time until the required group of three at that concentration has been completed.

trimmed and smoothed. The three hearts are picked up and the bottom of the strainer is touched to a clean glass plate to remove adhering Tyrode solution. The hearts are then put into the drop in the well slide and the stop watch started.

*Timing the appearance of end points.* The end point adopted was the appearance of a block between atrium and ventricle or of dropped beats for the whole heart. The former accounts for the great majority of cases. In making observations, however, caution should be exercised not to consider a block between ventricle and conus since this occurs earlier and with less consistency than A-V block. As a matter of fact, most of the conus may be cut off in the dissection if desired but great care should be used to maintain the integrity of the atrium. The time for the appearance of the end point is then recorded for each of the three hearts after which they are discarded. This process is repeated 16 times, making

TABLE 1  
*Observations for a typical assay*

Assay No. 38, January 1, 1942, temperature  $37.5 \pm 0.3^\circ$  C.

Unknown—Tincture prepared from commercial sample of powdered leaf, 0.1 gm. per cc. menstruum.

Standard—U.S.P. XI Reference powder, 0.1 gm. per cc. menstruum.

	$S_1$	$S_2$	$U_1$	$U_2$
Concentration, cc. tincture per 10 liters.....	4.17	12.50	8.33	25.00
Dilution.....	1:2400	1:800	1:1200	1:400
	8.42	3.00	8.88	1.50
	10.10	3.48	9.95	2.68
	7.98	3.89	5.98	3.10
	8.40	2.48	6.30	2.20
	7.35	2.60	7.20	3.00
	10.28	3.00	7.45	3.22
Block time in minutes. ....	5.10	2.60	5.70	2.40
	5.40	3.10	6.00	2.78
	7.00	4.20	6.63	2.82
	5.10	1.74	5.28	2.78
	7.14	3.00	6.70	3.00
	8.00	4.50	7.94	3.40

a total of 48 hearts, 24 on standard and 24 on unknown. A definite order should be adopted such as the following: low concentration of standard ( $S_1$ ); low concentration of unknown ( $U_1$ ); high concentration of standard ( $S_2$ ); high concentration of unknown ( $U_2$ ). The temperature in the warm chamber is recorded after every group of three and the data discarded if this is found to vary by more than  $0.3^\circ$  C. from the average temperature for that assay. A complete set of observations for an assay is given in table 1.

**RESULTS. Calculation of ratio of standard to unknown and its standard error.** The observations in table 1 have been so planned as to make use of the factorial coefficients devised by Bliss and Marks (3) for a "two dose assay." A complete set of calculations for these data is given in tables 2 and 3. Time and concen-

tration have both been changed to logarithms and the following conventional notation has been used:  $X$  is log concentration;  $x$  is a factorial coefficient<sup>5</sup> which is used as a deviation from an arbitrary mean log concentration of zero;  $\bar{X}_s$  and  $\bar{X}_u$  are mean log concentrations for standard and unknown respectively;  $y$  is log time;  $Y_p$  is the sum of the log time values for a single log concentration  $X$ ;  $S(xY_p)$  is the sum of the products of each factorial coefficient ( $x$ ) for a given log concentration and its corresponding  $Y_p$  (three values of  $S(xY_p)$  will thus be obtained from which variances (1), (2) and (3) in table 3 are calculated);  $N$  is the number of observations at each concentration;  $n$  is the total number of

TABLE 2  
*Calculations of observations given in table 1*

	$S_1$	$S_2$	$U_1$	$U_2$
$X = \log \text{concentration}$ . . . . .	0.6201	1.0060	0.9206	1.3979
	0.9253	0.4771	0.9484	0.1761
	1.0043	0.5416	0.9978	0.4281
	0.9020	0.5899	0.7767	0.4914
	0.9213	0.3945	0.7903	0.3424
	0.8663	0.4150	0.8573	0.4771
	1.0120	0.4771	0.8722	0.5079
$y = \log \text{time}$ . . . . .	0.7076	0.4150	0.7559	0.3802
	0.7324	0.4914	0.7782	0.4440
	0.8451	0.6232	0.8215	0.4502
	0.7076	0.2405	0.7226	0.4440
	0.8537	0.4771	0.8261	0.4771
	0.9031	0.6352	0.8998	0.5315
$Y_p$ . . . . .	10.3837	5.7776	10.0558	5.1500
$Sy = 31.3671$		$Sy^2 = 22.929,363$		
		$(Sy)^2/n = 20.497,812$		
		Total Sum of Squares	2.330,551	

observations;  $D^2$  and  $B^2$  are variances which are defined in table 3;  $s^2$  is the variance of the population;  $I$  is the interval in logarithms between concentrations  $S_1$  and  $S_2$  or  $U_1$  and  $U_2$  which must, of course, be identical. From table 3 it will appear that the log ratio of potencies ( $M$ ) is  $-0.2531 \pm 0.0342$ . The

<sup>5</sup> The appropriate coefficients for the possible sources of variation which may be isolated statistically are given in the third column of Table 3. The factorial notation may be equated to the familiar expressions of Fisher as follows:  $S(X - \bar{X})^2 = (I/2)^2 NS(x^2)$  and  $S(X - \bar{X})(y - \bar{y}) = (I/2)S(xY_p)$  where  $S(xY_p)$  is calculated using the row of coefficients for linear regression. The common slope is then,  $2S(xY_p)/INS(x^2)$  and its standard error is  $s_2/I\sqrt{NS(x^2)}$  which in this case reduces to  $S(xY_p)/24I \pm s_2/2I\sqrt{3}$  since  $N$  is always 12 and  $S(x^2)$  is always 4.

trimmed and smoothed. The three hearts are picked up and the bottom of the strainer is touched to a clean glass plate to remove adhering Tyrode solution. The hearts are then put into the drop in the well slide and the stop watch started.

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a total of 48 hearts, 24 on standard and 24 on unknown. A definite order should be adopted such as the following: low concentration of standard ( $S_1$ ); low concentration of unknown ( $U_1$ ); high concentration of standard ( $S_2$ ); high concentration of unknown ( $U_2$ ). The temperature in the warm chamber is recorded after every group of three and the data discarded if this is found to vary by more than  $0.3^\circ \text{C}$ . from the average temperature for that assay. A complete set of observations for an assay is given in table 1.

**RESULTS. Calculation of ratio of standard to unknown and its standard error.** The observations in table 1 have been so planned as to make use of the factorial coefficients devised by Bliss and Marks (3) for a "two dose assay." A complete set of calculations for these data is given in tables 2 and 3. Time and concen-

*Tests of significance.* The computations used here have been given only briefly since they have been described in detail by Bliss and Marks (3) using the assay of insulin as illustration. The factorial coefficients greatly facilitate the calculation of the potency ratio and its standard error and the three variance ratios given in table 3 upon which rest the determination of the validity of the assay. The significance of these variance ratios may be tested with the tables of Snedecor (4) or Fisher and Yates (5) using in each case 1 and 44 degrees of freedom for the greater and lesser mean squares respectively. The three variance ratios may be interpreted as follows:

( $F_1$ ): The ratio 1.97 fails to exceed the expected value of  $F$  for a probability of 0.05 and is therefore not significant. While it is desirable that standard and unknown be compared at equal time levels as was the case in this assay, it is not

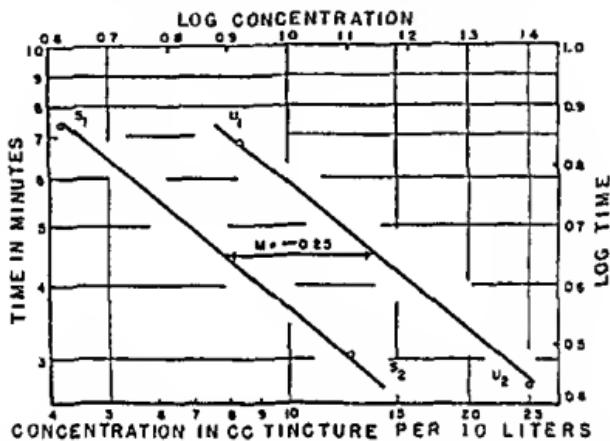


FIG. 3. GRAPH SHOWING THE TIME-CONCENTRATION CURVES FOR STANDARD ( $S_1, S_2$ ) AND UNKNOWN ( $U_1, U_2$ ) FOR THE ASSAY OF TABLES I, II AND III

The log ratio of potencies ( $M$ ) may be estimated by inspection of the graph; it is the average distance between the lines in a direction parallel to the axis of concentrations.

necessary to reject an assay for which  $F_1$  is significant as long as no mean block time falls outside the limits of 2.8 and 12 minutes.

( $F_2$ ): The value 195 exceeds greatly the expected  $F$  for a probability of 0.001. The assay should be rejected if  $F_2$  fails to be highly significant at these odds. Such an occurrence would indicate that either (1) the concentrations are so close together that the method will not differentiate them, or (2) the hearts were insensitive to the drug, or (3) the high concentrations fell along the floor of the curve.

( $F_3$ ): The variance due to lack of parallelism for the standard and unknown curves is here less than the error term and therefore not significant. Naturally the assay must be rejected if  $F_3$  exceeds the expected  $F$  and a probability of 0.05 would seem to be sufficiently exacting. The results of every assay were computed and tested for validity as in this example.

*Position of the floor of the curve.* The floor of the curve was explored with an

official tincture prepared from the U.S.P. XI Reference Powder. Six hearts were used on each of the following concentrations in cc. per 10 liters: 2.95,  $2.95\sqrt{2}$ ,  $(2)(2.95)$ ,  $(2\sqrt{2})(2.95)$ ,  $(4)(2.95)$ ,  $(4\sqrt{2})(2.95)$ , and  $(8)(2.95)$ . This scheme gives an equal logarithmic dosage interval of 0.1505. The mean log block time ( $y$ ) was plotted against log concentration ( $X$ ) in figure 4 and the floor of the curve is seen to occur at about 2.8 minutes. Anything lower than this time level must, accordingly, be avoided in an assay. The ceiling of the curve is indeterminate since the variation becomes excessive and the experiment unduly protracted.

*Linearity of the relationship between log time and log concentration.* Using the data from the experiment just described a rigid test was made for linearity within the 2.8 and 12 minute time limits, orthogonal coefficients being employed

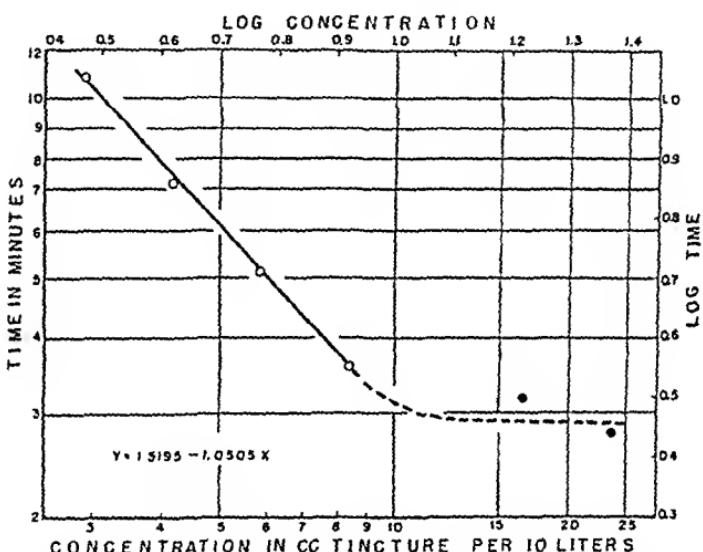


FIG. 4. GRAPH SHOWING THE TIME-CONCENTRATION RELATIONSHIP IN MORE DETAIL FOR THE ACTION OF DIGITALIS ON THE EMBRYONIC CHICK HEART

The portion of the curve to be avoided in an assay is indicated by the dotted line.

to simplify the computation. The method has been applied to bio-assay problems before (3) and it will be sufficient to give in table 4 the analysis of variance for the data. The large value of 123 for the variance ratio for linear regression as compared with the ratios for a second or third order polynomial makes it clear that a linear relationship is the best interpretation of the data.

*Magnitude of the slope and standard deviation.* The slopes have been found to vary to a considerable extent from day to day and the hearts seem more sensitive in the summer than in the fall and winter. Thus, in table 5 the first group of six assays carried out in the summer shows a variation in slope from 0.6 to 1.5, while the second or winter group is 0.5 to 0.8. However, this does not constitute any overall difference in precision since the standard deviations listed in table 5 are also about twice as large in summer as in winter. The two effects

balance each other since the standard error of the potency ratio is directly proportional to the standard deviation and inversely proportional to the slope. It might be noted here that because of the wide seasonal variation in slope the use of a standard curve would not be valid. Thus, it is essential that at least two concentrations be used on both the standard and unknown so that the slopes are determined independently for each assay.

*Influence of temperature.* The influence of temperature was explored in the hope of thereby increasing the precision. An experiment was carried out using an official tincture prepared from U.S.P. XI Reference Powder. Two appropriate concentrations were tested at  $35.0^{\circ} \pm 0.3^{\circ}$  C. and two more at  $40.0^{\circ} \pm 0.3^{\circ}$  C. with twelve hearts at each concentration. The data for the two temperatures were obtained in parallel on the same lot of eggs much as in an assay. The results are shown graphically in figure 5. It is evident that the slope was

TABLE 4

*Analysis of variance as a test of linearity for the relationship between log time and log concentration*

	VARIATION DUE TO	DEGREES OF FREEDOM	SUM OF SQUARES	VARIANCE	VARIANCE RATIO (F)
1	Linear regression of log time (y) on log concentration (X)	1	0.740,031	0.740.031	123
2	Regression of y on X according to a second order polynomial	1	0.000,647	0.000.647	1.07
3	Regression of y on X according to a third order polynomial	1	0.000,195	0.000.195	<1
4	Experimental error or the discrepancy between items 5 and items 1 + 2 + 3	20	0.121,913	0.006.005	1
5	Total	23	0.872,656		

doubled by a  $5^{\circ}$ C. rise in temperature. However, the standard deviation was not improved at the higher temperature and the hearts beat at an abnormally high rate. A more physiologic temperature of  $37.5^{\circ}$ C. was therefore adopted. This experiment serves in addition to emphasize the importance of accurate temperature control during an assay.

*Breed of hen.* Eggs from the two principle types of hen, Leghorns and Rhode Island Reds, were used in various assays over a short period of time and no significant difference in slope or standard deviation was observed.

*Practical considerations.* On the basis of about 50 assays the yield from a lot of eggs was roughly as follows: 73% satisfactory as in figure 2B and 27% rejected because of, abnormalities (0.7%), sterility (7%), underdevelopment as in figure 2A (12%), over-development as in figure 2C (8%). Incubation of nine dozen eggs assured 48 hearts for the assay with enough in excess to take care of

TABLE 5  
*Homogeneity of replicate assays*

DATE OF ASSAY	STANDARD DEVIATION $\sqrt{\frac{1}{2}}$	COMBINED SLOPE $\pm$ STANDARD ERROR	GRAMS U.S.P. XI REFERENCE POWDER EQUIVALENT TO ONE TABLET OR ONE GRAM POWDER $\pm$ STANDARD ERROR
Preparation: Lederle Lot 18 Digitalis Tablets			
June 19, 1941.....	0.117	-1.10 $\pm$ 0.09	0.0880 $\pm$ 0.0062
June 28, 1941.....	0.168	-1.51 $\pm$ 0.13	0.0710 $\pm$ 0.0054
June 30, 1941.....	0.154	-1.35 $\pm$ 0.17	0.0835 $\pm$ 0.0061
July 4, 1941.....	0.114	-0.78 $\pm$ 0.14	0.0703 $\pm$ 0.0069
July 5, 1941.....	0.113	-0.96 $\pm$ 0.11	0.0775 $\pm$ 0.0061
July 1, 1941.....	0.131	-0.61 $\pm$ 0.15	0.0696 $\pm$ 0.0111*
Weighted mean ( $\bar{M}$ )			0.0773 $\pm$ 0.0027
$\chi^2_M$ with 5 degrees of freedom			6.720
Preparation: New York Heart Association Powder No. 7			
December 3, 1941.....	0.088	-0.68 $\pm$ 0.06	0.665 $\pm$ 0.058
Deeember 12, 1941.....	0.097	-0.83 $\pm$ 0.07	0.592 $\pm$ 0.046
January 21, 1942.....	0.065	-0.56 $\pm$ 0.04	0.599 $\pm$ 0.048
January 23, 1942.....	0.076	-0.64 $\pm$ 0.04	0.740 $\pm$ 0.058
January 28, 1942.....	0.062	-0.55 $\pm$ 0.04	0.709 $\pm$ 0.053
January 30, 1942.....	0.084	-0.66 $\pm$ 0.04	0.756 $\pm$ 0.064
Weighted mean ( $\bar{M}$ )			0.662 $\pm$ 0.022
$\chi^2_M$ with 5 degrees of freedom			8.891

\* No selection of hearts according to degree of maturity was exercised and the standard error will be seen to be nearly double the usual value.

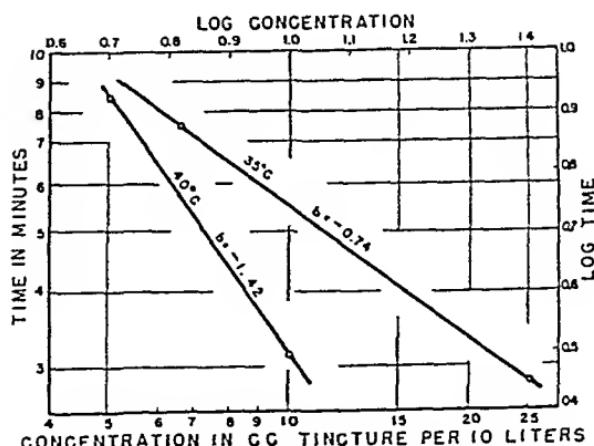


FIG. 5. GRAPH SHOWING THE INFLUENCE OF TEMPERATURE ON THE SLOPE OF THE TIME-CONCENTRATION CURVE

orienting experiments. The cost of the embryonic chick heart assay is of the same order as the cat and the frog, with the advantage that fertile eggs can be readily obtained in all seasons and districts. Each assay requires the attention of one person for about five hours.

*Precision and accuracy.* The reproducibility of the method may be judged from table 5 in which are listed the results of six replicate assays on each of two preparations. It will be seen that the potency ratios and their standard errors appear to be consistent within each group. As an objective test of homogeneity  $\chi^2$  was calculated as suggested by Miller, Bliss and Braun (6) and in each case failed to reach a probability level which would cast doubt on the randomness of the observations. The weighted mean potency ratio ( $\bar{M}$ ) was also determined

TABLE 6  
*Assay of known dilutions of digitalis tincture*

DATE OF ASSAY	GRAMS U S P XI REFERENCE POWDER EQUIVALENT TO 1 CC DILUTED TINCTURE $\pm$ STANDARD ERROR		PERCENT ERROR
	Known value	Found by assay	
January 14, 1942	0.0333	0.0355 $\pm$ 0.0031	6.0
November 11, 1941	0.0572	0.0610 $\pm$ 0.0062	6.6
January 17, 1942	0.0750	0.0775 $\pm$ 0.0060	3.3

TABLE 7  
*Grams U S P XI Reference Powder equivalent to one gram of unknown  $\pm$  standard error*

	NEW YORK HEART ASSOCIATION POWDER NO. 7	POWDER A	POWDER B
Cat	0.61* $\pm$ 0.05	1.10† $\pm$ 0.06	0.78† $\pm$ 0.04
Man	0.67* $\pm$ 0.12	0.78† $\pm$ 0.03	0.86† $\pm$ 0.05
Embryonic chick heart	0.66† $\pm$ 0.02	0.69 $\pm$ 0.07	0.56 $\pm$ 0.04

\* Results of Dr. B. J. Vos, Jr. and Dr. Lloyd C. Miller, Food and Drug Administration, on normal man.

† Results of Dr. Harry Gold, Cornell University Medical College, on cardiac patients.

‡ Weighted mean of six assays (see table 5).

(6) for each of the series using the invariancee ( $1/s^2_{\text{v}}$ ) as the weight for each ratio. In the assay dated July 1, 1941 (table 5) the selection of hearts according to degree of maturity was intentionally omitted. The standard error was accordingly about twice the usual value, which demonstrates the advantage of as rigid a selection as possible. As a test of the accuracy of the method three dilutions were made of an official tincture of the U.S.P. XI Reference Powder and blind assays were then run on each using its parent tincture as standard. The results are given in table 6 in which the errors will be seen to lie within 3 and 7% of the known values. For all assays, the standard errors are on the average  $\pm 8\%$  of the potency ratios which would seem to make the method comparable in accuracy to other bio-assays. It is recognized, of course, that the only true

test of accuracy lies in the satisfactory prediction of the human potency ratio for two preparations of different origin. At present only preliminary data can be included. In table 7 is given a comparison of the results obtained on the cat, embryonic chick heart and man for three samples of digitalis powder. It will be seen that the deviations between the human and cat assays are respectively 9, 41 and 9%, while for the same preparations by the chick heart they are 2, 12 and 35%.

#### SUMMARY AND CONCLUSIONS

1. A technique has been described for the assay of digitalis tincture or whole leaf preparations which requires no elaborate equipment and no extensive training on the part of the operator.
2. A design has been suggested which facilitates the calculation of the potency ratio of standard to unknown with its standard error and appropriate tests of significance.
3. The results of replicate assays have been found to be consistent within their sampling errors.
4. The error in the assay of known dilutions of tincture was found in three cases to lie within 7%.
5. Comparative assays of three digitalis powders using the U.S.P. XI Reference Powder as standard suggest that the chick heart method gives results which are at least as close to man as the cat assay. Further work, of course, is necessary to reach a definite conclusion on this point.

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# EFFECTS OF COCAINE AND SYMPATHOMIMETIC AMINES ON HUMORAL TRANSMISSION OF SYMPATHETIC NERVE ACTIONS

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In a previous paper (1), it was pointed out that certain sympathomimetic amines, notably ephedrine and tyramine, which produce vasoconstrictor (pressor) effects and are desensitized by cocaine *in vivo*, were almost completely ineffective when dissolved in Locke's solution and perfused through cats' legs. The addition of 1:10,000,000 epinephrine to the Locke's solution restored the vasoconstrictor activity of the amines, as did also perfusion with desibrinated ox-blood. The latter has been shown to contain appreciable amounts of epinephrine for several days after collection (2). Perfusion with other agents such as barium chloride, post-pituitary solution, calcium chloride, insulin, thyroxin, and gelatin (to simulate blood viscosity) in Locke's solution failed to restore the vasoconstrictor activity in the absence of epinephrine. This dependence of the actions of ephedrine and tyramine on available epinephrine was explained according to current theories of blocking by these amines of ferments which normally destroy epinephrine in the peripheral structures and thus permit its accumulation in greater than (local) threshold concentration (Gaddum *et al.*).

The desensitization of the pressor effects of tyramine and ephedrine by cocaine could be explained by the same theories if cocaine also blocked these ferments *in vivo* as it has been shown to do *in vitro* (8, 11). That is, after complete cocaineization, ephedrine and tyramine could cause no further interference with the destruction of the tissue epinephrine supply. Such enzymic inhibition would explain the sensitization of epinephrine effects on blood pressure by cocaine and by these same amines, phenomena which have been known for some time. Proof of such a mechanism for ephedrine has already been obtained by Gaddum and Kwiatkowski (3). It therefore seemed desirable to determine whether cocaine also prevents the destruction of epinephrine in tissues, and hence acts fundamentally similarly to the sympathomimetic amines. The literature on this phase of cocaine actions has recently been thoroughly reviewed (12) and need not be recapitulated here. Of special interest in this connection is the report of Clark and Raventos (4) that cocaine does not increase the amount of sympathin liberated by nerve stimulation, but rather slows the rate of sympathin inactivation in tissues. If this is true, perfusion with a weak solution of epinephrine together with cocaine should show a higher recovery of epinephrine in the perfusate than perfusion with epinephrine alone.

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The results obtained by us in support of this proposition are clear-cut, although not as extensive as we would ordinarily desire. Since opportunity for continuing work on this problem seems unlikely in the near future, the results are presented herewith in their present form.

#### RATE OF EPINEPHRINE DESTRUCTION

Cats were anesthetized with sodium pentobarbital, the femoral arteries cannulated and perfused with Locke's solution to wash the blood out of the legs. The limbs were severed through the hip joint and placed in a warm water-jacketed funnel from which the venous outflow was collected quantitatively. The femoral cannula was joined to a perfusion pump of constant output, and with a mercury manometer which traced arterial pressure changes on a kymograph as the peripheral resistance altered. This was the equivalent of blood pressure tracings *in vivo*. The Locke's perfusion fluid was warmed to 37°C., and the rate of flow was kept constant throughout, after adjustment at the outset, by changing the rate or stroke of the pump to give a satisfactory level of pressure. As a further check the rates of flow were measured continuously through timed samples of the output fluid. The epinephrine content of both output and input fluid were measured in samples taken at frequent intervals, by means of Shaw's method (5). From these values we computed the total amount of epinephrine destroyed per minute by the vascular bed of the leg in the presence and absence of cocaine. When appropriate blank and control samples were run, it was not found necessary to use the adsorption steps of Shaw's procedure, since interfering substances usually removed in this way were not a complicating factor in our perfusions with Locke's solution. Control estimations showed that epinephrine could be measured quantitatively by this method in the presence of cocaine as high as 1:4000 concentration, which was much more than was used in the perfusions. Therefore, cocaine did not interfere with the chemical estimations of epinephrine.

Perfusion with Locke's solution alone caused a slight initial vasodilatation and then stabilization of perfusion pressure at a lower than normal level. Addition of 1:150,000 to 1:500,000 cocaine hydrochloride to the Locke's solution produced no vasoconstriction, but, on the contrary, a vasodilatation, thereby demonstrating a lack of direct pressor power. Epinephrine in concentrations of 1:20 to 1:5 million in the Locke's solution caused an elevation of pressure. This was accompanied by a rate of epinephrine destruction that was fairly constant for each animal and of a magnitude roughly proportional to the epinephrine present in the perfusion fluid. The addition of cocaine, 1:500,000 to 1:150,000, to the epinephrinized perfusion fluid caused additional rises in pressure (fig. 1). The rate of epinephrine destruction was decreased at the same time. Subsequent perfusion with Locke's solution alone caused a fall in pressure to the original control level. The leg was still quite reactive to injected epinephrine at the conclusion of each experiment. The essential parts of a pressure tracing are presented in figure 1, and the numerical results are summarized in table 1. Table 1 shows that the average rate of destruction of epinephrine was 1.02 micrograms per minute. This was reduced to 0.60 microgram per minute when cocaine was added to the perfusion fluid. Therefore, there was an overall decrease of epinephrine destruction of about 40%. The average pressor response to epinephrine was increased from 26 mm. in the controls to 47 mm. when cocaine was added. These results therefore substantiate the theory that cocaine interferes with destruction of epinephrine by the tissues.

EFFECT OF SYMPATHETIC NERVE DEGENERATION. The humoral transmission theory implies that the sympathetic nerve endings play little rôle in vasomotor reactions, except as sources of humoral agents in response to nerve stimulation. It therefore became of interest to determine whether degeneration of these

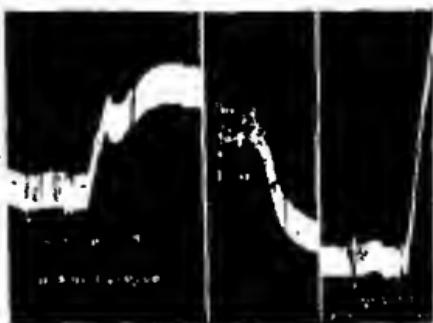


FIG. 1. PRESSOR RESPONSES IN A PERFUSED CAT'S LEG WHEN EPINEPHRINE ALONE OR EPINEPHRINE AND COCAINE TOGETHER ARE ADDED TO THE LOCKE'S PERfusion FLUID

TABLE 1

*Rate of epinephrine destruction and pressor effects in perfusion of cats' isolated hind legs with Locke's solution containing epinephrine and cocaine*

CONCENTRATION		EPINEPHRINE DESTROYED IN MICROGRAMS PER MINUTE			PRESSURE RISE OVER CONTROL LEVEL IN MM. HG.	
Epinephrine	Cocaine	During perfusion with epinephrine	During perfusion with epinephrine and cocaine	Difference	During perfusion with epinephrine	During perfusion with epinephrine and cocaine
1:20 M		0.28			10	
1:20 M	1:500 T	0.33	-0.34	0.67	16	29
1:10 M	1:500 T	0.63	0.46	0.17	10	13
1:10 M	1:250 T	0.40	0.40	0.23	10	17
1:10 M	1:500 T	1.19	1.04	0.15	68	104
1:10 M	1:150 T	0.94	0.74	0.20	12	25
1:5 M	1:350 T	2.02	1.33	0.69	26	96
Average	...	1.022	0.605	0.417	26.4	47.3

M = millions; T = thousands.

endings would modify the responses to the amines in a way which would be consistent with the proposed humoral mechanisms.

The effects of sympathectomy, and of complete degeneration of the nerves, upon the pressor potencies of epinephrine and four other sympathomimetic amines were determined by the same perfusion method, with *ox blood* as the perfusion fluid. This blood was defibrinated and diluted with an equal volume of Locke's solution. It was kept in a refrigerator for at least 24 hours to destroy the vasotonin (1). Since it still possessed much of its original epinephrine content (2), the responses which required the presence of this substance could be studied without addition of the hormone. The hind legs of the cats were sympa-

thectomized by excision, through a median abdominal incision, of the sympathetic chains on both sides from under the crurae of the diaphragm to below the brim of the pelvis, and avulsion of the nerve trunks from regions in which they could not be reached directly (7). The completeness of the sympathectomy was confirmed at subsequent necropsy. An average period of 35 days was allowed for complete degeneration, no animals being used earlier than the fourteenth day after the preliminary operation.

Since ox blood had not been used previously by us as a perfusion medium for cats' legs, control determinations with it of the pressor ratios of cobefrine, neosynephrine, ephedrine, and tyramine against epinephrine as a standard were made, as described before (1), on 10 normal unsympathectomized cats. These values were in good agreement with those observed when the cat's own blood was used for the perfusion. Then we redetermined the ratios in 6 sympathectomized preparations, using identical perfusion technic and ox blood as the perfusion fluid. The average ratios and their standard errors are shown in table 2. The absolute pressor responses to epinephrine were approximately doubled by the sympathectomy, in accordance with usual experience. This sensitization to epinephrine is presumably the result of increased permeability of the smooth

TABLE 2

*Effect of sympathetic degeneration upon the pressor ratios to epinephrine in perfusion of isolated hind legs of cats with defibrinated blood*

	COBEFRINE	NEO-SYNEPHRINE	EPHEDRINE	TYRAMINE
Control (not sympathectomized) . . .	{ 6.0± 0.47	8.2± 1.48	178± 52.0	60.3± 16.7
Sympathectomized . . .	{ 6.8± 1.73	8.4± 2.57	412± 116	199± 23.0

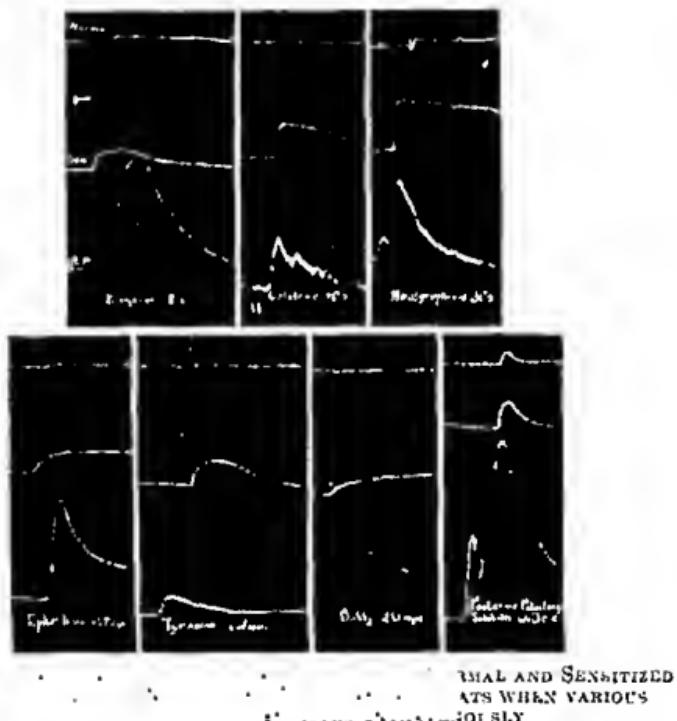
muscle cell membrane, which permits epinephrine to diffuse more readily from innervated to non-innervated cells, and thereby to produce increased actions (6).

However, the data in table 2 show that the pressor potencies of cobefrine and neosynephrine also were increased, and to the same degree, so that the ratios of activity to epinephrine were unchanged within the limits of expected variability. Thus cobefrine and neosynephrine were affected qualitatively and quantitatively like epinephrine, which lends support to our previous suggestion (1) that these amines act fundamentally like epinephrine, or "sympathin," and differ mainly in requiring a higher dosage. Other evidence of their sympatheticotropic action has been available for some time, such as the observations of Tainter and Stockton of sensitization by cocaine of neosynephrine (9) and those of Jang (10) of cobefrine by ephedrine.

With ephedrine and tyramine, however, the *absolute* potency was practically unaltered by the sympathetic degeneration, and these compounds therefore had a decreased potency relative to epinephrine. These results proved that these latter two compounds did not act through the sympathetic nerve endings of blood vessels, since these had been removed by the process of degeneration.

The failure to demonstrate a sensitized response was further evidence of a fundamental difference in their mechanism of action from epinephrine.

**SENSITIZED NICTITATING MEMBRANE.** Since the cat's nictitating membrane has been widely used for studies in this field, it was thought that observations on this preparation would afford comparison with another type of smooth muscle. Accordingly, the effects of the same amines were determined on the normal and denervated nictitating membrane of the cat. The superior cervical ganglion was removed on one side of each of 6 cats, and 12 to 35 days were allowed for degeneration. Each cat was then anesthetized, decerebrated, and its adrenals



The upper tracing is of the normal and the middle of the sensitized membranes; the lower one shows blood pressure.

tied off, so the results would not be complicated by epinephrine secretion. The carotid artery was cannulated for recording blood pressure, and the two nictitating membranes were connected to delicately balanced levers with exactly equal leverage and tension, so that the responses on the two sides and from animal to animal could be quantitatively compared. Injections of the drugs in appropriate doses were made through the femoral veins. A typical tracing is shown in figure 2. About 50 injections of the various drugs were made into these 6 cats, and the rise of the levers carefully compared on the sensitized and normal sides. The average results are summarized in table 3. The absolute magnitude of the differences on the two sides was not reliable for all the drugs, since the doses

of some, while producing a definite response on the sensitized side, were below the threshold on the normal side, and, hence, the full potential difference in response was not recorded.

The observations revealed a phenomenon which has not been taken into account by a few who have used this membrane as a test object for experimentation on mechanisms of sympathetic actions. That is, the increase in permeability produced by sympathetic degeneration in this membrane also permits musculotropie drugs, such as barium and post-pituitary extract, to penetrate into the cell more readily, or at least to produce enhanced responses. This sensitization is not altered significantly by complete atropinization, so that parasympathetic reactions probably are not involved. Therefore, unless it can be clearly shown by other evidence that a given drug is not a direct smooth muscle stimulant, increased responses in the sensitized nictitating membrane cannot be used as

TABLE 3

*Comparison of the average contraction of cat's nictitating membranes on the sympathectomized and normal sides in response to various pressor drugs*

	EPINEPHRINE 0.5-1.0 7/K	COCAINE 40-50 7/K	NEPHRINE 50-60 7/K	EPHEDRINE 40-100 7/K	TYRAMINE 1-500 7/K	POST VITIARY SOLUTION 0.2-0.3 CC/K	BARIUM CHLORIDE 6-20 MG/K
	Mm.*	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
Normal.....	4.3	0.7	2.0	0.8	0.6	4.3	3.0
Sensitized (sympathectomized).....	12.3	4.0	13.3	6.2	7.0	9.7	4.3
Increase in constriction.....	8.0	3.3	11.3	5.4	6.4	5.4	1.3

\* Mm. is the average millimeter rise of the lever from contraction of the nictitating membranes.

proof of sympatheticotropic action, since such might occur equally well with a purely muscular stimulant.

This point is important for the correct interpretation of the total actions of ephedrine and tyramine, both of which increased the contraction of the sensitized nictitating membrane. Since both ephedrine and tyramine can increase the activity of atropinized intestine under appropriate conditions, these amines probably also possess some musculotropie actions in addition to their sympathetic effects. Therefore, quantitative experiments on the nictitating membrane with these drugs would be difficult to interpret, and the conclusions would not necessarily be transferrable to other organs. Therefore, our observations on this tissue were not extended.

#### SUMMARY AND CONCLUSIONS

1. When cocaine and epinephrine in Locke's solution are perfused together under constant conditions through the vasicular bed of the cat's isolated hind leg, the pressor responses are increased, and the rates of destruction of epinephrine by the tissues are decreased, in comparison to those when the perfusion fluid contains epinephrine alone.

2. When the sympathetic nerves to the leg are allowed to degenerate and the perfusion carried on with diluted defibrinated ox blood, the absolute pressor potency of epinephrine, eobefrine, and neosynephrine is increased several fold, but in approximately the same ratio; hence, their relative activity is unaltered, as compared with parallel ox blood perfusions of unsympathectomized legs. However, perfusion with ephedrine and tyramine reveals that these amines are substantially unaltered in their absolute vasoconstrictor potency, either by the absence of the sympathetic endings or by the state of sensitization, and therefore, these latter amines act independently of the sympathetic endings in the blood vessels.

3. The cat's nictitating membrane is unsuited for this type of study requiring sympathetic degeneration, since this process, in this tissue at least, causes simultaneous sensitization to musculotropic drugs, such as post-pituitary solution and barium chloride, as well as to the sympathetic agents.

4. These various results are compatible with, and lend further support to, current theories of humoral transmission of sympathetic nerve impulses to blood vessels. According to these concepts, ephedrine and tyramine, and now cocaine, partially block the ferment within the smooth muscle cell which inactivates the epinephrine being taken into the cell continuously from the blood stream, or the sympathin being derived intermittently from the local sympathetic nerve endings. The resulting local increase in the epinephrine or sympathin concentration gives rise to the characteristic pressor response. It is suggested here that previous cocaineization prevents the pressor actions of ephedrine and tyramine by blocking the ferment through which they normally act, and by the same means sensitizes the responses to sympathetic nerve stimulation and injection of epinephrine. Degeneration of the sympathetic nerves renders the smooth muscle cells more permeable, so that epinephrine penetrates more quickly, thereby building up a higher concentration within the cells, causing an increased response. Cobefrine and neosynephrine act similarly although they require higher doses. *The increased permeability from sympathetic degeneration does not sensitize the responses of blood vessels to ephedrine and tyramine, indicating again a difference in their mechanism of action from sympathicotropic agents.*

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# DISTRIBUTION OF EXOGENOUS BROMINE IN CATS AFTER TREATMENT WITH TRIBROMOETHANOL, TRIBROMOACETALDEHYDE AND TRIBROMOACETIC ACID

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In a preliminary study of distribution of bromine in cats treated with various narcotics containing bromine (1), large differences between tribromoethanol (avertin) and tribromoacetaldehyde (bromal) were noted. The present report amplifies observations of these differences and extends the study to include tribromoacetic acid. A more complete picture of the relations between these compounds could be obtained if distribution of bromoform were also estimated, but the method of assay used yields recoveries of only some 35% of bromoform while over 95% of the other three agents are recovered. In view of the current interest in halogenated fatty acids (2, 3), however, a report on distribution of tribromoacetic acid is justified.

It was suggested previously (1) that distribution studies may be of value in predicting possible pathologic side-effects of new agents, since good correlation was obtained between relative tissue affinities and the reported pathologic side-effects of the older agents studied. This information may be of more immediate value than extensive studies of pathologic effects on normal animals, for many of the serious clinical side-effects of new agents occur rarely and then perhaps only in patients with disease of an organ having high affinity for the agent.

Inasmuch as apparent tissue affinities may be misleading when overwhelming doses of agents are given, it is desirable to use amounts comparable to those used clinically. The sensitive and accurate test for tissue bromine devised by Brodie and Friedman (4) has made possible the estimation of distribution after small doses of agents containing bromine have been given.

**METHODS.** Fifteen young, healthy adult cats were treated intramuscularly with the three agents, and 7 more were used as untreated controls. Doses were equivalent in bromine content to 0.2 gm./kg. for all agents. Dissections were made 60 minutes after injection and the order of removal of organs was the same as in previous work (1). Diet and other care of the cats were unchanged and other necessary precautions were observed as before. Groups of cats were approximately evenly divided as to sex.

Of the 15 treated cats, 5 were given avertin, 4 were given bromal and 6 received tribromoacetic acid. Avertin was used as the commercial solution of tribromoethanol in amylenic hydrate. Bromal was obtained from the Eastman Kodak Co. Tribromoacetic acid was prepared from bromal and was neutralized immediately before use with  $\text{NaHCO}_3$ . One cat in each of the groups treated with bromal and tribromoacetate received the agent in solution with an amount of amylenic hydrate equivalent to that contained in an avertin solution of the same molar concentration. The volume of injected fluid was made in each case to 20 cc. with physiologic saline prepared from reagent  $\text{NaCl}$  containing less than 11  $\gamma$  of Br and

I together in the amount used. None of the solutions was warmed to more than 40° in preparation and all were injected at 38° into the thigh muscles.

The assay method of Brodie and Friedman (4) was used for estimating the bromine content of tissues, with the minor modifications developed by Abreu (5). Some 3000 determinations have now been made with this method in this and previous studies.

**RESULTS.** In cats treated identically with the same agent, amounts of bromine found in blood or tissues of individual animals often differ by 20% from the appropriate mean values. There is, however, little variation in the relative distribution of "exogenous" bromine in the tissues, among cats after treatment with the same agent. That is, if increments in the bromine content of tissues are expressed in percent of the increase in bromine content of the blood in each instance, results are consistent within each group of treated cats. It may be concluded that amounts absorbed vary from individual to individual with any one agent but that the relative distribution of these amounts to the various tissues is fairly constant. It may also follow that the undetermined factors governing absorption from the site of injection do not necessarily affect the characteristic affinities of different tissues or the manner of removal of the agent from the circulation by the tissues.

Results are therefore expressed in figure 1 as the mean percent of exogenous bromine in tissues in terms of the exogenous bromine content of blood, which in each instance is arbitrarily given the reference value of 100%. The mean result for each type of tissue is obtained by correcting the individual total bromine concentration by the appropriate mean concentration found for the corresponding tissue of 7 untreated cats, calculating this corrected value as percent of the corrected value for blood of the individual treated cat, then averaging these percent values for the same tissue in all cats treated with the same agent. These calculations are based on results of analyses expressed as  $\gamma/\text{gm.}$  of tissue, and "total bromine concentration" signifies the sum of "exogenous" and "intrinsic" bromine concentrations, not the absolute amount of bromine in an entire organ.

Total concentrations of bromine in tissues of cats treated with avertin or bromal, and of untreated cats, have been presented previously (1) for individual animals. With tribromoacetate, the degree of variation of total bromine concentrations of tissues is comparable to that found with the former two agents and consequently these detailed results are not given here.

Figure 1 presents graphed data concerned with the distribution of exogenous bromine in tissues after treatment. Each bar represents the mean value found for a tissue, in terms of percent of the exogenous bromine concentration of the blood. From left to right, the bars represent mean concentrations for blood (100%), plasma, 5 parts of the central nervous system, 5 glands, 7 muscles, bile, urine, spleen, skin, lung, and red marrow from the femur.

The mean exogenous bromine content of blood after treatment may be interpreted as a rough index of absorption. Increases in bromine content of blood above the mean control level of 20  $\gamma/\text{gm.}$  were: for avertin, 154; for bromal, 167; and for tribromoacetate, 362  $\gamma/\text{gm.}$  That is, the mean exogenous bromine concentration of the blood was about 8% greater in cats treated with

bromal and about 135% greater in cats treated with tribromoacetate than in cats treated with avertin. It is apparent that distribution of these agents is

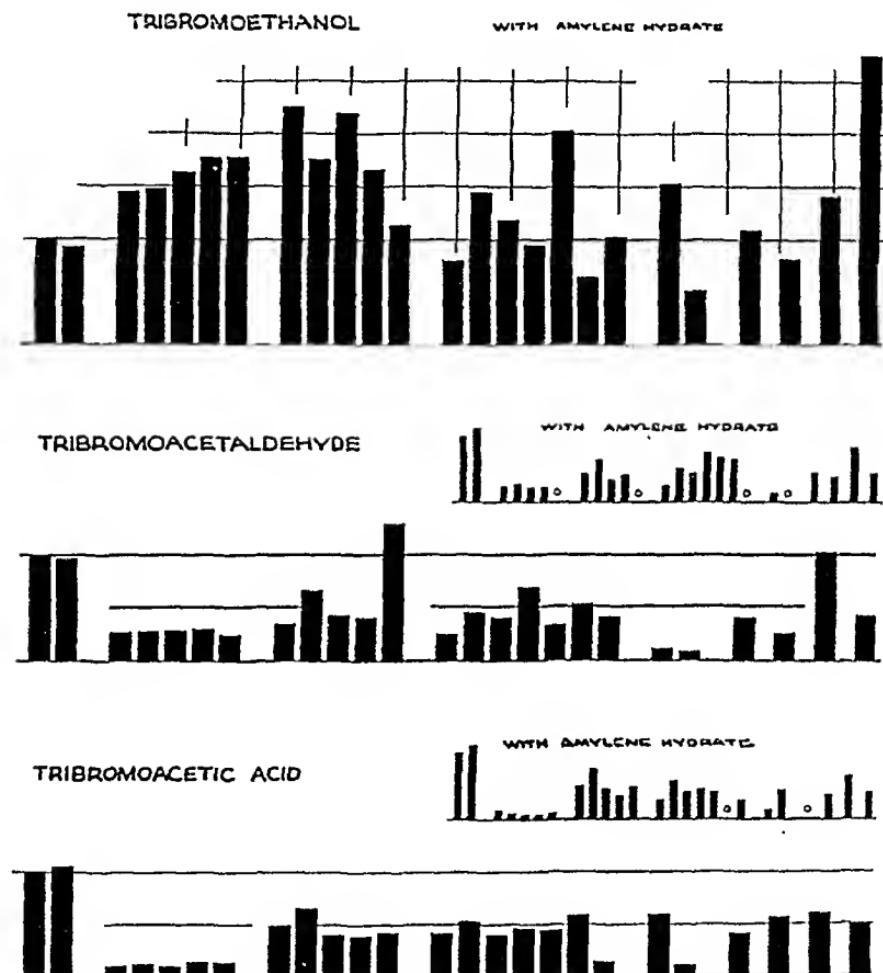


FIG. 1. DISTRIBUTION OF EXOGENOUS BROMINE IN TISSUES OF CATS TREATED WITH AVERTIN, BROMAL AND TRIBROMOACETATE

Bars represent mean concentrations in tissues, expressed as percent of mean concentration in blood. See text.

From left to right, bars represent values for blood (100%), plasma, cerebral cortex, cerebellum, basal ganglia, medulla, cervical cord, liver, kidney, adrenal, pancreas, testes, abdominal muscle, myocardium, jejunum, bladder, stomach, uterus, seminal vesicles, bile, urine, spleen, skin, lung, and red marrow from the femur.

Insets (graphs with small bars) represent values for tissues in the same order, of cats treated with amylen hydrate in addition to bromal or tribromoacetate. Tissues omitted are noted as "O".

tissues is related to the nature of the compounds and is not an artifact dependent upon amounts absorbed, even though no truly quantitative measure of absorp-

tion may be implied from the blood concentrations because of the wide differences in affinities of tissues for the different agents.

Addition of amylen hydrate to solutions of bromal or trihromoacetate does not influence distribution of these substances, as illustrated in the small graphs inset in Figure 1. The presence of amylen hydrate in the solution of avertin presumably is not responsible for the differences in distribution of bromine after treatment with this agent from that in cats treated with bromal or trihromoacetate. Although bromine content of blood was less in both cats treated with added amylen hydrate than in the corresponding series of cats treated without amylen hydrate, it cannot be concluded that amylen hydrate significantly hinders absorption. Increments in bromine content of the blood were 143  $\gamma$ /gm. in the cat treated with bromal + amylen hydrate and 280  $\gamma$ /gm. in the cat treated with trihromoacetate + amylen hydrate. Neither of these concentrations varies from the corresponding mean concentrations by three times the standard deviations of these means.

**DISCUSSION.** The present method of assay permits only the estimation of total bromine concentration in tissues. There is thus no direct evidence that the data here reported represent the true distribution of the compounds given, in unchanged form. However, four considerations support the view that these data may be so interpreted. The distribution of bromine after treatment with avertin is markedly different from the distribution of NaBr previously reported (1) and there are also significant differences between the distribution of NaBr and of bromine after treatment with bromal or trihromoacetate. The agents used are not highly unstable *in vitro* under conditions approaching those in tissues, although a small amount of decomposition occurs in basic solutions maintained at 40° for 60 minutes or more. Even if appreciable degradation does occur within tissues, distribution of the resultant bromide or other decomposition products will be determined in part by the primary distribution of the agents before hydrolysis or oxidation occurs. Finally, the compounds exhibit their characteristic physiologic effects, which differ from those of inorganic bromides, throughout the experimental period. Hence, it is felt that the data signify at least a close approximation of the true distribution, and that any decomposition is sufficiently slight to be neglected. For brevity, the following discussion assumes that this is so, but it is based solely on estimates of total bromine present in tissues after treatment.

Avertin is distributed in all tissues except striated muscle, skin, uterus and bladder in concentrations greater than that in the blood. Significantly larger concentrations occur in brain, glands, bile, heart, lungs and marrow than in blood. Contrariwise, bromal and trihromoacetate are distributed in smaller concentrations in all tissues than in blood, except for similar concentrations in lungs and testes of cats treated with bromal. The distribution of the latter two agents roughly resembles that of inorganic bromide.

With the doses employed, cats treated with avertin or bromal are narcotized, but several tissues in these treated animals have a higher content of exogenous bromine than does the brain. Trihromoacetic acid is not narcotic in the dose

given and all tissues show a greater affinity for this substance than does the brain.

All of the agents are excreted in the urine and bile to some extent. A higher content appears in bile than in urine. Much larger amounts of exogenous bromine are excreted in cats treated with avertin than in cats treated with tribromoacetate and relatively little is excreted by cats receiving bromal. Biliary secretion of bromal and tribromoacetate is not promoted by simultaneous administration of amyleno hydrate. Quantitative comparison of rates of excretion may not be made from the data available since samples for assay were necessarily diluted by pre-formed urine or bile present in the respective reservoirs prior to injection of the agents.

Further study of analogous series of compounds must be made before various generalizations concerning the relation of chemical structure and physical properties to partition of the agents between blood and plasma, to distribution in other tissues, and to apparent differences in rates of absorption, which are strongly suggested by the present data, can be seriously considered. This and previous work (1) show, however, that high affinities of the central nervous system toward different agents containing bromine have so far been found only for those lipolytic narcotics which have proven clinically useful, although with the majority of these, tissues other than the brain have even higher affinities. In relation to the present findings, it may be noted that bromal is not considered to be a useful narcotic agent in higher animals.

#### SUMMARY

Distribution of exogenous bromine in tissues of cats is described after treatment of these animals with tribromoethanol, tribromoacetaldehyde and tribromoacetic acid. Avertin is distributed in most tissues in greater concentration than in blood, while the reverse holds for the latter two agents. The central nervous system has a high affinity for avertin but not for bromal and tribromoacetic acid. Several tissues other than brain exhibit higher affinities for each of the agents than does the brain. Absorption of any one agent is variable but the relative distribution of absorbed amounts is constant.

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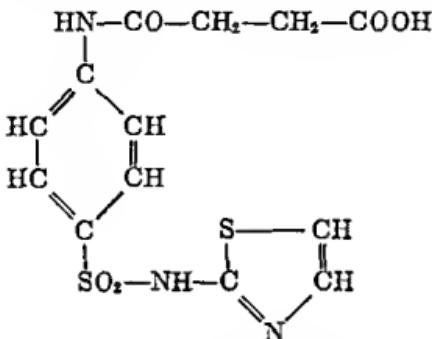
# A TOXICOLOGICAL STUDY OF SUCCINYL SULFATHIAZOLE<sup>1</sup>

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A series of  $N^4$ -dicarboxylic acid-substituted sulfonamides has been synthesized by Moore and Miller (1). One of this series, 2-( $N^4$ -succinyl-sulfanilamido)-thiazole, or succinyl sulfathiazole, was reported by Poth and Knotts (2) to exert a marked bacteriostatic action on the coliform organisms of the intestinal tract of



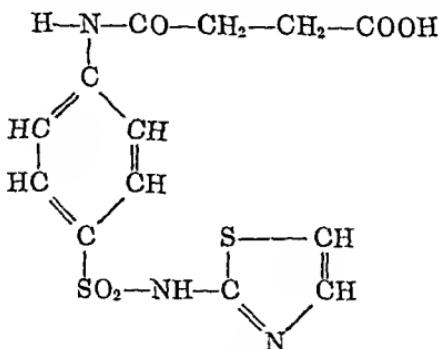
dogs. These workers encountered no toxic manifestations when the drug was administered orally every 4 hours, in doses up to 1 gram per kgm. per day, for a period of five weeks. During the experimental period, the dogs gained weight and no evidence was found of either gross or microscopic tissue damage. The striking action of the drug on the intestinal flora was evidenced by the reduction in the coliform count of the feces from an average normal of 10,000,000 per gram to less than 100 per gram. Semi-fluidity and loss of odor were manifestations of the profound alteration in the character of the feces. From the low blood concentration (average: 1.5 mgm. % free drug (as sulfathiazole) and 2.0 mgm. % combined drug (as succinyl sulfathiazole)) and the small amount excreted in the urine (approximately 5% of the ingested drug) it is evident that the compound is absorbed to a very limited extent from the gastro-intestinal tract of dogs.

In the preliminary report of Poth and Knotts, mention was made of the use of the drug in a series of 40 human subjects. A more extensive use of succinyl sulfathiazole in a larger series of human patients has been described in subsequent communications (3, 4). Encouraging results have been obtained in the preparation of the bowel for surgery, and preliminary results of interest in bacillary dysentery are reported.

A series of experiments is described in this communication in which succinyl sulfathiazole was administered orally to mice, rats and monkeys in order to

<sup>1</sup> Sharp and Dohme has applied its trade-mark 'Sulfasuxidine' to this new sulfonamide.

obtain more complete data on the acute and chronic aspects of the toxicity of the drug. Experiments are also described in which the drug was used under the most severe conditions, lacking any resemblance to its probable therapeutic use. It was found that the drug can be given in doses which are relatively large in



### Succinyl Sulfathiazole

comparison with those of the well-known sulfonamides, without producing indications of toxic manifestations. In fact, in order to obtain any toxic or lethal effect, it was found necessary to administer succinyl sulfathiazole by routes other than oral, and in doses many times larger than those likely to be required in therapeutic use.<sup>2</sup>

**EXPERIMENTAL.** *Acute toxicity. Oral.* We have been unable to kill mice (Carworth CF<sub>1</sub>) with succinyl sulfathiazole by oral administration. Single doses as high as 26 grams per kgm. have been given orally, in the form of a 33% suspension in 0.5% tragacanth; the resultant mean blood concentration (5 mice), two hours after intubation, was 2.1 mgm. % free drug (sulfathiazole) and 3.2 mgm. % combined drug (as succinyl sulfathiazole).<sup>3</sup> Doses of 40 grams of the

<sup>2</sup> Succinyl sulfathiazole occurs as a white, practically odorless and tasteless crystalline powder, which is soluble in water at 37°C. to the extent of about 70 mgm. per 100 cc.; it melts with decomposition at 192-195°C. The drug is a strong acid and dissolves in an aqueous solution of sodium bicarbonate with effervescence to form the soluble sodium salt. Sodium succinyl sulfathiazole is very soluble in water; a 50 per cent, neutral, aqueous solution is stable at 10°C. for 10 to 14 days.

<sup>3</sup> Application of the usual method (5, 6) for the determination of the so-called "free" component of total sulfonamide derivatives in blood, urine, etc., gives a measure of the concentration of sulfathiazole which, to a limited extent, is split off from succinyl sulfathiazole by the tissues.

Determination of the concentration of the "total" sulfonamides, in this case "free" sulfathiazole, plus unhydrolyzed succinyl sulfathiazole, plus any other derivatives in which substitution of the para-amino group has occurred (e.g., acetylsulfathiazole), requires special treatment. The blood filtrate, or other material, must be heated for about two hours at 100°C. in the presence of approximately 1.5 normal sodium hydroxide (5 parts of filtrate plus 1 part of 40% sodium hydroxide) in order to insure complete liberation of the sulfathiazole component. With the aid of phenolphthalein the excess of alkali is neutralized with hydrochloric acid (4*N*); and excess of acid is added to give a concentration, after the final volume adjustment, of 0.2 normal. Diazotization and color formation are then carried out in the usual manner.

Since these experiments were carried out a simpler "acid" method for the determination

drug per kgm. have been given in the form of a 50% suspension in 15% acacia; the mean blood concentration produced (5 mice) was 2.6 mgm. % sulfathiazole, and 4.2 mgm. % succinyl sulfathiazole. Following such dosage mice show no abnormalities in appearance or activity other than those seen in controls which are intubated with the same volume of the suspending medium.

*Intraperitoneal* administration of succinyl sulfathiazole was accomplished by the olive oil suspension method which Marshall *et al.* (7) used in the determination of the toxicity of sulfaguanidine for mice. Succinyl sulfathiazole failed to kill mice (Carworth CF<sub>1</sub>) when given intraperitoneally in doses as large as 3.5 grams per kgm. suspended in 0.5 cc. of olive oil; the LD<sub>50</sub> was found to be approximately 5.7 grams per kgm. The results of experiments in which this method was used are shown in table 1. Using sulfaguanidine, Marshall *et al.* (7) found 0.5 gram per kgm. to kill 11 of 15 mice; the mean blood concentration

TABLE 1

*Intraperitoneal toxicity in mice of succinyl sulfathiazole suspended in olive oil*

DOSE IN grams/kgm.	NUMBER DEAD/TOTAL	MORTALITY PERCENTAGE	APPROXIMATE TIME OF DEATH IN HOURS	MEAN BLOOD CONCENTRATION 2 HOURS FOLLOWING INJECTION	
				Free*	Combined*
3.5	0/30	0		6.1	185
4.8	4/15	27	5.0	6.1	101
5.5	12/30	40	5.0	8.2	283
6.0	16/25	64	3.5	7.3	232
7.0	27/30	90	3.5	10.0	269
8.0	15/15	100	3.5	11.4	378

\* See footnote.\*

found at death following this dosage was 16.3 mgm. % "free" and 36.1 mgm. % "total."

The sodium salt of succinyl sulfathiazole in aqueous solution was injected intraperitoneally in Swiss mice. The toxicity of the drug in this form appeared to be somewhat less than that of the free acid in oil suspension, but a lethal effect was produced much more rapidly (30-60 minutes). The LD<sub>50</sub> of the aqueous solution of the sodium salt was approximately 7.5 grams per kgm.; the mean blood concentrations produced by the LD<sub>10</sub> dose, 7.0 grams per kgm., 30 minutes

nation of "total" sulfanamides has been developed. To the filtrate sufficient hydrochloric acid is added to make the concentration about 1N; after heating at 100°C. for two hours, and volume readjustment, diazotization may be carried out without neutralization of the excess acid.

The amount of so-called "combined" drug is obtained by calculation from the "total" and "free" values. In this communication the values for "total" and "combined" forms of the drug are expressed as succinyl sulfathiazole. The concentration of "free" drug in the blood is usually low; as a result the amount of "combined" drug which occurs as acetyl-sulfathiazole would also be low. A close approximation to the true situation in the blood probably results from the consideration of all "combined" drug as succinyl sulfathiazole.

after injection, were: sulfathiazole, 9.7 mgm. % and succinyl sulfathiazole, 775. mgm. %.<sup>4</sup>

*Chronic toxicity. Rats.* A group of 10 animals (Sunny Hill strain) was given free access to a powdered commercial diet containing 5 per cent succinyl sulfathiazole; a control group of 10 received the basal diet *ad libitum*. The changes in weight during a period of 33 days are shown as follows:

	ORIGINAL WEIGHT	INCREASE IN WEIGHT
	grams	grams
Control group.....	113 ± 12.5	98 ± 19.0
Succinyl sulfathiazole group.....	115 ± 13.0	106 ± 11.5

The average blood levels produced by feeding succinyl sulfathiazole to rats under these and other dietary conditions are shown in table 2.

TABLE 2

*Concentration of sulfonamides in the blood of rats receiving succinyl sulfathiazole as a dietary constituent*

DIET	PER CENT SUCCINYL SULFATHIAZOLE IN DIET	MEAN BLOOD CONCENTRATION	
		Free <sup>‡</sup>	Combined
S-1*	1	1.3 ± 0.2	4.6 ± 1.1
S-1	5	2.4 ± 0.2	14.0 ± 7.2
Chow†	5	1.4 ± 0.1	4.6 ± 2.0

\* Diet S-1 consists of casein, 18; sucrose, 76; salts (U.S.P. #2), 4; corn oil, 2; choline chloride, 0.2; thiamine hydrochloride, pyridoxine hydrochloride and riboflavin, of each, 0.003; calcium pantothenate, 0.002; and nicotinic acid, 0.0025.

† Chow—a commercial pulverized ration (Wayne).

‡ See footnote.<sup>3</sup>

During the experiment there was no evidence of toxicity. Histological examination was made of specimens of liver, kidney, spleen, bone-marrow, pyloric portion of the stomach, duodenum, ileum and sciatic nerve obtained from both control and experimental animals. No abnormalities were found.

Recently Mackenzie *et al.* (8) described an interesting pathological change in the thyroid glands of rats fed 1 or 2 % sulfaguanidine for a period of 4 weeks.

\* The results obtained following injection of the aqueous solution of sodium succinyl sulfathiazole should not be compared too closely with those obtained following injection of succinyl sulfathiazole suspended in olive oil, for the following reasons: (1) a different strain of mice was used; (2) when a lethal effect was produced by the aqueous solution, about 30-60 minutes were required, therefore blood samples were taken at 30 minutes rather than at 2 hours; (3) dosage with oil suspensions is less accurate because of the difficulty in obtaining an exact weight-volume relationship. It is believed that no great significance is to be attached to the apparent difference between the toxicity of the drug suspended in oil and that of the sodium salt in aqueous solution.

On the 2% sulfaguanidine diet the thyroid glands were hyperemic and 3 to 4 times larger than the glands of the control animals. Rats fed a diet containing 5% succinyl sulfathiazole for a period of 43 days disclosed no hyperemia or hypertrophy of the thyroid; the glands of the experimental rats were indistinguishable from those of the controls.

*Monkeys. Technique of Experiment.* Nine monkeys (*Macaca mulatta*) were stomach-tubed at 4 hour intervals, day and night, for a period of 30 days. Six of these animals were given succinyl sulfathiazole in the form of a neutral solution of the sodium salt:<sup>4</sup> two received 0.5 gram per kgm.; two, 1.5 grams per kgm.; and two, 5.0 grams per kgm. The volume of each injection was 15 cc.; the three control animals were given concurrent intubations with water. In addition each of the nine animals was given 3 cc. of a yeast concentrate<sup>5</sup> once daily. Twice daily the monkeys were given a varied diet consisting of fresh fruits and vegetables, whole wheat bread, and raw peanuts.

At the beginning of the experiment and during its course, determinations were made of the erythrocyte count, total and differential leucocyte counts, and the concentration of hemoglobin and of plasma proteins.<sup>6</sup> Blood concentrations of the drug, both free and combined, were determined at frequent intervals. The concentration of drug in the urine was determined as frequently as uric specimens, uncontaminated with feces, could be obtained without catheterization.<sup>7</sup>

With the aid of sterile instruments a sample of feces was taken from the rectum of each monkey at frequent intervals. These samples were examined bacteriologically by Dr. W. F. Verwey, who has kindly permitted us to include his results in this paper.

Throughout the experiment appetite was consistently good, and evidence of depression, nausea, or other abnormality was not seen at any time. Regurgitation, although infrequent, was always associated with the stimulation incident to the withdrawal of the stomach tube. Diarrhea was seen only once during the experiment. On the twelfth day one of the monkeys on the 5 grams per kgm. per day dosage had a fluid stool, slightly tinged with blood. This condition was almost certainly due to injury resulting from manipulation during the collection of a stool specimen on the preceding day, since recovery occurred within 24 hours and no further diarrhea or bloody stool was seen in this animal. In all other animals the stools were essentially normal in appearance, possibly somewhat

<sup>4</sup> The solution of sodium succinyl sulfathiazole was prepared from the desired amount of the drug by the addition of water and sodium hydroxide (sodium bicarbonate may also be used) until the required volume and pH were attained. The sodium salt of succinyl sulfathiazole has a markedly different chemical structure from that of the sodium salts of the usual heterocyclic sulfonamides, since in sodium succinyl sulfathiazole it is the succinyl portion of the molecule which is involved in salt formation.

<sup>5</sup> The approximate analysis of the yeast concentrate (3 cc.) is as follows: thiamin hydrochloride, 1.8 mgm.; riboflavin, 0.6 mgm.; nicotinic acid, 0.7 mgm.; pyridoxine hydrochloride, 0.3 mgm.; pantothenic acid, 1.3 mgm.

<sup>6</sup> Plasma protein was determined by the method described by Barbour and Hamilton (9).

<sup>7</sup> The contamination of the skin and fur of the monkeys, as well as of the cages, with feces containing large amounts of sulfonamides, rendered the collection of "pure" samples of urine very difficult. The tendency of monkeys to urinate during the excitement of being caught offered a further obstacle to the successful collection of urine samples. Frequent catheterization is undesirable and difficult, particularly in the male, which sex included all but one of the experimental animals.

softer and lighter in color than were those of the control monkeys. The condition of the monkeys in such toxicity studies can be gauged roughly by the energy with which they resist being caught, held and intubated; all nine animals were as vigorous during and at the end of the experiment as they were at its inception. All monkeys showed an increase in weight at the end of the experiment; the controls gained 0.2, 0.5 and 0.7 kgm. respectively, while the gain in weight of the experimental animals varied from 0.5 to 0.7 kgm.

In figure 1 are shown the results of several determinations of the erythrocyte count, the concentration of hemoglobin and of plasma protein. It will be seen that throughout the period of administration of sodium succinyl sulfathiazole the results of the determinations of hemoglobin concentration and erythrocyte count fell within the normal limits of variation. The plasma protein levels appear to be somewhat lower than normal on the last day of the experiment, but it is unlikely that such lowering is significant since the value for one of the controls is as low as that of any experimental animal. The mean value of 45 determinations on 24 normal monkeys was 8.2 grams of protein per 100 cc. of plasma, with a standard deviation of 0.8 grams and maximum and minimum values of 10.27 and 5.58 grams.

In table 3 are presented the results of determinations of the concentrations of free and combined drug in the blood of monkeys receiving sodium succinyl sulfathiazole orally. Blood samples (0.05 cc.) were taken from the ear 4 hours following dosage; however, when the interval was reduced to 2 hours higher blood levels were not obtained.

From the low blood concentrations found it is clear that this drug is either absorbed very poorly from the gastro-intestinal tract or removed very rapidly from the blood following absorption. All available data indicate that both factors operate in maintaining the concentration of sulfonamides in the blood at a low level when succinyl sulfathiazole is administered. Consideration of the sulfonamide content of blood and urine obtained from the monkeys on the present experiment clearly indicates that absorption of succinyl sulfathiazole is very limited in this species. Experiments will be described subsequently in which it is shown that parenterally injected sodium succinyl sulfathiazole is very rapidly excreted by the kidney.

Comment has been made previously concerning the difficulty of obtaining urine entirely free from fecal (and therefore from drug) contamination. The contamination of metabolism cages with feces containing the drug precluded the possibility of collecting 24 hour urine specimens in which the sulfonamides were derived solely from the kidneys. Recourse was made, therefore, to calculations based on the analyses of those wholly uncontaminated specimens of urine which were obtained and on the determinations of the maximum volume of urine excreted during 24 hour periods.

The average concentration of drug in the urine of the animals on the highest dosage of succinyl sulfathiazole (5 grams per kgm. per day) was 15.4 mgm. % sulfathiazole and 46.1 mgm. % succinyl sulfathiazole. The maximum concentration found in the urine at any time was 24.1 mgm. % sulfathiazole and 75.6

mgm. % succinyl sulfathiazole. The volume of urine excreted by the monkeys usually amounted to between 200 cc. and 400 cc. per day; the largest amount

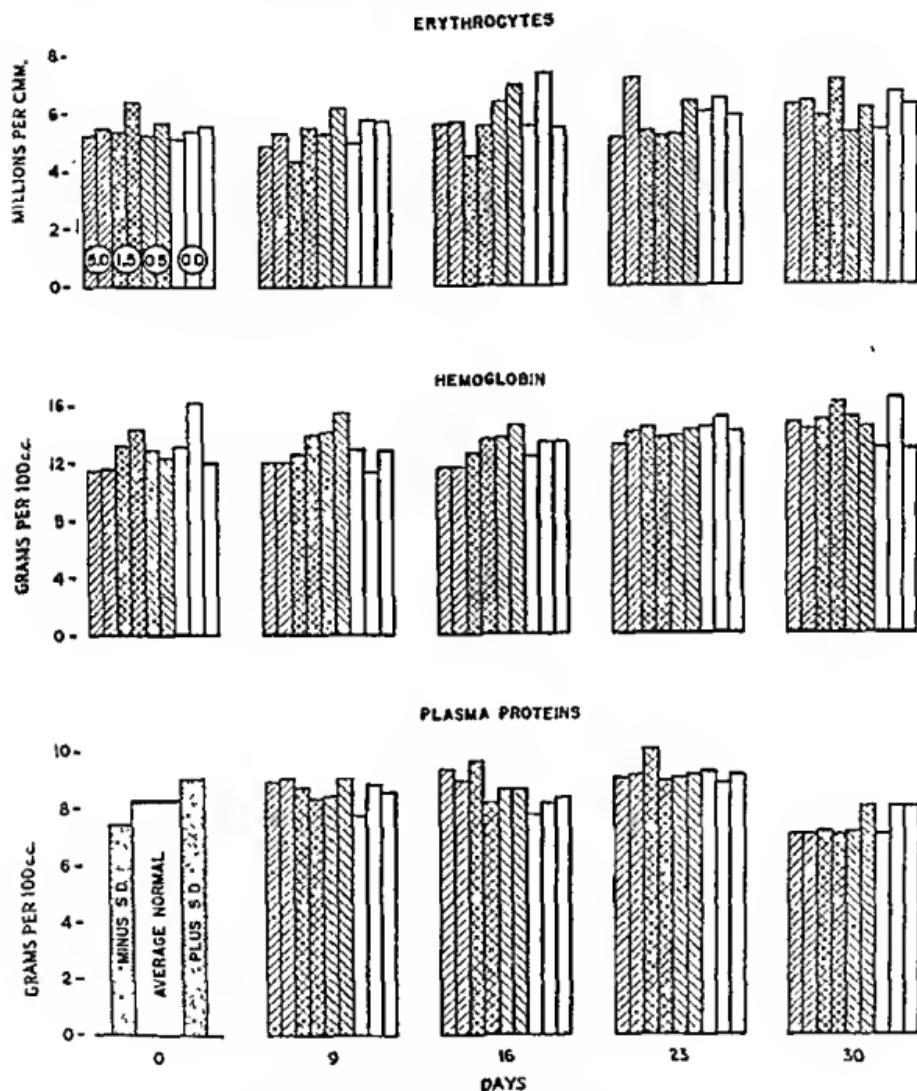


FIG. 1. OBSERVATIONS MADE ON THE BLOOD OF MONKEYS DURING THE ORAL ADMINISTRATION OF SODIUM SUCCINYL SULFATHIAZOLE

apparently excreted was 600 cc. From the maximum figures given above it may be estimated that the maximum sulfonamide output per day did not exceed

145 mgm. of sulfathiazole and 455 mgm. of succinyl sulfathiazole, together equivalent to about 660 mgm. of the ingested drug. The heavier of the two animals on the highest dosage received 19.6 grams of succinyl sulfathiazole per day; therefore, the maximum amount of drug which appeared in the urine did not exceed 4% of the total ingested dose in these monkeys.

At the completion of the experiment the drug-treated animals and two of the three controls were sacrificed and subjected to autopsy. No demonstrable gross lesions were found in any of the monkeys. Sections of lung, heart, stomach, intestinal tract, pancreas, spleen, liver, kidney, adrenal, ureter, urinary bladder, gall bladder, lymph node and bone-marrow were taken for microscopic study. Histological examination of these tissues revealed no abnormalities, with the exception of certain greenish-brown pigment deposits in the lung. These deposits were seen in the controls as well as in the treated animals, and no significance can be attached to their presence. Microscopic examination of the

TABLE 3

*Concentration of sulfonamides (in mgm. per 100 cc.) in the blood of monkeys following the oral administration of succinyl sulfathiazole as the sodium salt*

DAYS	5.0 GRAMS PER KGM.				1.5 GRAMS PER KGM.				0.5 GRAMS PER KGM.			
	Monkey A		Monkey B		Monkey A		Monkey B		Monkey A		Monkey B	
	Free*	Comb.*	Free	Comb.	Free	Comb.	Free	Comb.	Free	Comb.	Free	Comb.
1	1.0	3.8	0.8	4.2	0.8	1.8	1.0	1.9	0.5	1.7	0.8	1.8
2	0.8	2.6	0.8	3.9	0.5	2.8	0.8	2.4	0.5	2.4	0.5	2.4
6	0.4	3.1	0.3	3.3	0.4	3.0	0.4	3.2	0.5	3.2	0.3	2.4
9	0.8	4.0	0.5	3.2	0.5	2.9	0.5	2.6	0.2	2.4	0.2	1.8
16	0.7	2.6	0.5	2.0	1.1	3.5	0.8	2.4	0.4	0.4	0.9	2.9
23	0.8	3.1	0.6	2.8	0.5	2.1	0.5	5.3	0.1	2.2	0.2	2.5
30	1.3	2.2	1.0	1.8	0.6	1.3	0.4	0.6	0.3	1.1	0.2	1.3
31†	0.2	1.0	0.1	1.0	0.2	0.8	0.3	1.3	1.3	1.4	0.4	1.1

\* See footnote.\*

† Approximately 18 hours following last dosage.

sediment from urine specimens taken from the bladder at autopsy also showed no abnormal constituents.

BACTERIOLOGICAL STUDIES OF FECAL SPECIMENS FROM MONKEYS ON CHRONIC TOXICITY TEST. Prior to the administration of succinyl sulfathiazole several fecal samples were taken from all animals. These, and specimens taken from the three controls during the experiment (a total of 65 samples), were studied bacteriologically as a basis for comparison of the results following the use of succinyl sulfathiazole. Identification procedures indicated that the predominating organisms, under the dietary conditions of the experiment, were *not* coliform bacteria, but lactobacilli, streptococci and anaerobic bacilli.<sup>9</sup> The average

<sup>9</sup> Bacterial counts were made on aerobic and anaerobic blood agar plates and by the highest dilution of fecal solids producing growth in horse meat infusion broth and Brewer's thioglycollate medium. The coliform content of fecal specimens was determined by counts of desoxycholate agar pour-plates. All media contained para-aminobenzoic acid (5 mgm. %).

coliform count was only 100,000, while total counts ranged from 100 million to 10 billion.<sup>10</sup> No effect on the total bacterial count of the feces was exerted by the drug at any time.<sup>11</sup>

Poth and Knotts (2) found succinyl sulfathiazole to exert a marked effect on the fecal coliform count of dogs on a meat diet; with dosages of 1 gram or less per kgm., coliform counts of 10 million were reduced to 100 or less. In monkeys, reduction of the coliform count to 100 or less was produced by the drug, although with less rapidity and consistency, and only with higher dosage than is required in the dog (and apparently in the human being (2, 3, 4)). Two monkeys, receiving 0.5 gram of succinyl sulfathiazole per kgm. per day in six divided doses, had coliform counts of 100 after 4 and 9 days respectively, but the reduction was not constant; subsequently, counts as high as 10,000 alternated with low counts. With higher dosages the results were more definite and more consistent. The coliform counts of two animals on 1.5 grams per kgm. daily were depressed to 100 in 4 and 6 days, respectively. Of all coliform counts made from the onset to the conclusion of this dosage, 57 per cent were 100 or less. On 5.0 grams per kgm., 70 per cent of all fecal specimens had coliform counts of 100 or less; this reduction occurred after 2 and 3 days, respectively.

#### PARENTERAL ADMINISTRATION OF SODIUM SUCCINYL SULFATHIAZOLE IN UNILATERALLY NEPHRECTOMIZED MONKEYS.

*Technique of experiment.* The evidence so far presented indicates that succinyl sulfathiazole is poorly absorbed from the gastro-intestinal tract of mice, rats and monkeys. In the event, however, that extensive absorption might occur, under conditions at present unpredictable, determinations were made of the effect of high blood concentrations. Parenteral injections were made in monkeys (*Macaca mulatta*) from each of which one kidney had been removed in order to restrict the ability of the animals to excrete the drug.

Under anesthesia produced by the intravenous injection of a thiobarbiturate, one kidney and a section of the liver were removed from each of six animals. These tissues served for the histological study of the pre-treatment state of the kidney and liver. Two to three weeks later, when the animals appeared to be in excellent condition, parenteral injections of succinyl sulfathiazole were begun. These were given daily for a period of 10 days, in a dosage of one gram per kgm. in the form of a 25 per cent solution of the sodium salt of the drug. One animal was given intraperitoneal injections; two were given the drug intravenously (1 cc. per minute); a fourth was injected subcutaneously, in four different sites, with equal portions of the daily dosage, a fifth received similar injections intramuscularly, while the sixth animal served as an operated control.

<sup>10</sup> All counts referred to are based on dilutions of one cc. (or one gram) of fecal solids.

<sup>11</sup> It would be exceedingly difficult to detect a change in the total bacterial count of the feces when organisms susceptible to the drug constitute a relatively small proportion of the total bacterial flora of the intestine. This does not imply that *E. coli* is the only organism which is affected by succinyl sulfathiazole. In this study, as well as in that of Poth and Knotts (2), attention was given to those normal inhabitants of the intestine which will grow on a desoxycholate medium. Preliminary results obtained by Poth (private communication) are indicative of the marked effectiveness of the drug in the therapy of bacillary dysentery. Poth and Knotts (4) state, "The Shiga, Flexner and Sonne strains of the dysentery bacillus are especially susceptible to the antibacterial action of this compound. The drug has no apparent effect on the growth of the typhoid and paratyphoid organisms, alpha Streptococcus faecalis or *Bacillus proteus*. *Bacillus aerohacter aerogenes* is resistant than *B. coli* to the action of succinyl sulfathiazole."

Prior to and at the conclusion of the experiment, determinations were made of the erythrocyte count, total and differential leucocyte counts, concentration of hemoglobin and of plasma protein. Attention was also given to the ability of the monkeys to remove isoiodidekon from the blood stream during a period of 30 minutes; the method employed was that of Graham and Cole as described by Elman and Heifetz (10). The concentration of the drug in the blood was determined at frequent intervals throughout the experiment.

During the first and tenth days of the period of parenteral injections, several urine samples were collected from each animal and analyzed for total sulfonamide content.

*Results.* During the injections the only definite evidence of possible toxic reactions were: a single instance of vomiting following an intravenous injection in one monkey (on the 8th day), lassitude for 5 to 10 minutes following a prolonged intravenous injection (and this was seen but twice), and lassitude following the intraperitoneal injections of the first and second days. That the general effect of the parenteral injections was not severe is indicated by the fact

TABLE 4

*Determinations of erythrocytic count, hemoglobin concentration, plasma protein concentration and isoiodidekon excretion in monkeys before and after a ten-day period of daily parenteral injections of sodium succinyl sulfathiazole (1 gram per kgm.)*

MONKEY*	ERYTHROCYTES IN MILLIONS PER CMM.		HEMOGLOBIN GRAMS/100 CC.		PLASMA PROTEIN GRAMS/100 CC.		PERCENTAGE EXCRETION OF ISOIODIDEKON IN 30 MINUTES	
	0 days		10 days		0 days		10 days	
I.V.	5.32	4.85	14.7	10.8	7.07	6.86	94	96
I.V.	5.39	5.31	13.6	13.1	7.89	7.86	91	
I.P.	4.33	4.37	14.8	11.1	7.07	7.35	92	92
I.M.	5.87	4.04	11.7	8.5	7.50	6.70	93	97
S.C.	5.18	3.96	13.9	8.9	7.58	6.42	92	95
Control	5.40	6.07	13.2	15.9	8.00	7.96	94	93

\* Each monkey is designated by the manner of parenteral injection: I.V. = intravenous; I.P. = intraperitoneal; I.M. = intramuscular; S.C. = subcutaneous.

that weight loss either did not occur in the treated animals or did not exceed that of the control, namely, 0.1 kgm.

Certain local effects were produced by the injection of the hypertonic solution (25%) of sodium succinyl sulfathiazole. Sclerosis of the veins of the intravenously injected monkeys interfered with the ease of administration after the first few days. Following subcutaneous and intramuscular injections, there was evidence of considerable discomfort. Damage at the injection sites was found at autopsy in the intramuscularly injected animal; this was evidenced microscopically by areas of recent hemorrhage, occasional tiny areas of necrosis and occasional nests of small round cells. Recent hemorrhages were found at the sites of subcutaneous injections, but no evidence was seen of an infectious process.

The data presented in table 4 indicate that a moderate fall in the concentration

of hemoglobin was produced in one of the intravenously injected monkeys; however, a negligible change occurred in the red cell count and hemoglobin concentration of a second animal subjected to identical treatment. The intraperitoneally injected monkey showed no depression in the erythrocyte count, but a probably significant decrease in hemoglobin concentration occurred. The intramuscularly and subcutaneously injected animals showed a clear-cut decrease in the number of red cells and definite depression of the hemoglobin concentration. No changes of significance were found in the leucocyte picture and no nucleated red cells were observed in the blood smears stained for differential leucocyte counts.

Microscopic studies (see below) of the tissues showed active bone-marrow in the control and more marked activity in each of the experimental animals. The bone-marrow of the intramuscularly injected monkey showed definite hyperplasia of the erythrocytic series. Although studies of the bilirubin concentration in the blood were not carried out, it is probable that blood destruction was caused in some of the experimental animals, with a resultant compensatory hyperplasia of the bone-marrow. Hemoglobinuria was not seen, and the occurrence of frank blood in the urine was noted only occasionally during the excretion of urine heavily loaded with micro-crystals of succinyl sulfathiazole following the parenteral administration of the drug.

Only in the case of the intramuscularly and the subcutaneously injected monkeys was there any appreciable depression of the plasma protein concentration (table 4), and this is of doubtful significance. The results of the isoiodoleikon excretion test, as carried out by us, suggest that no depression of this phase of liver activity occurred as a result of the drug therapy.

In table 5 data are presented on the concentration of sulfonamides attained in the blood following the parenteral administration of sodium succinyl sulfathiazole. When these data are examined in connection with those on the urinary excretion of the drug (table 6), it will be seen that the very high blood levels obtained by parenteral administration result in the rapid elimination of the drug by the kidney, with exceedingly high concentrations in the urine, e.g., 12 grams per cent. It is interesting to note that although the highest blood concentrations were obtained following intravenous injections, the rate of elimination by the kidney appeared to be significantly more rapid following intramuscular and subcutaneous injections.

The intensity of crystalluria apparently did not depend directly upon the concentration of drug in the urine. Although determinations of urinary pH were not made, the insolubility of the acid form of the drug indicates that the fundamental cause for precipitation is urinary acidity. At autopsy no evidence was found of crystals or concretions in any portion of the urinary tract.

Pathological examination of the tissues revealed no significant lesions that were not demonstrable in the control. The vascularity of the glomeruli in the experimental animals was somewhat greater than that of the control, and the activity of the bone-marrow was more marked in the monkeys receiving parenteral injections of drug, particularly the intramuscularly injected animal. Both

of these findings can be attributed to normal physiological responses of a compensatory nature. Four of the six animals, *including the control*, showed evidence of lymphoid hyperplasia, hyperemia of the adrenal medulla, tiny areas of necrosis in the liver, some desquamation of tubular epithelium of the residual kidney, and some pulmonary round cell infiltration. These changes may have been in part due to tuberculosis, although the animals had a negative tuberculin reaction at the beginning of the experiment. There is an interesting resemblance

TABLE 5

*Concentration of sulfonamides (in mgm. per 100 cc.) in the blood of unilaterally nephrectomized monkeys following the daily parenteral administration of sodium succinyl sulfathiazole (1 gram per kgm.)*

DAY	HOURS FOLLOWING INJECTION	I.V.*		I.V.		I.P.		I.M.		S.C.	
		Free†	Total†	Free	Total	Free	Total	Free	Total	Free	Total
1	0.2	3.5	152	4.1	170	—	74.5	—	136	—	65.1
	0.5	3.5	120	3.3	119	—	60.4	—	122	—	84.7
	1	2.7	104	2.9	98.0	—	44.7	—	93.3	—	91.0
	2	2.1	78.4	2.7	62.3	—	22.9	—	40.8	—	57.1
	4	2.3	32.3	2.7	22.3	—	—	—	—	—	—
	8	0.9	8.5	0.9	6.9	—	8.2	—	12.5	—	10.4
	24	0.5	6.3	0.9	7.5	—	8.2	—	7.8	—	7.2
3	4	—‡	26.7	—	28.5	1.0	62.3	0.6	36.1	1.4	74.6
	24	—	15.1	—	—	—	—	—	6.0	—	9.4
7	4	—	39.8	—	40.8	—	—	—	—	—	—
8	4	—	—	—	—	—	52.1	—	38.0	—	55.2
10	0.2	4.5	167	4.3	138	—	—	—	—	—	—
	0.5	2.9	136	4.8	171	4.2	150	2.8	141	2.9	133
	1	2.4	102	3.0	125	2.9	115	2.7	128	2.8	138
	2	1.8	68.4	2.3	74.2	2.5	99.3	2.0	94.1	2.1	103
	4	1.2	25.4	1.5	25.4	1.7	53.0	1.1	38.9	1.4	49.9
	8	—	10.4	—	8.8	0.8	6.9	—	7.2	1.1	9.4
	24	—	4.7	—	3.5	—	8.8	—	8.8	—	6.3

\* The designation of each monkey in the table corresponds with that of table 4.

† See footnote.‡

‡— Very faint color (less than 0.5 mgm. per 100 cc.).

of some of the lesions to the pathological changes which Sabin and collaborators (11, 12) described in monkeys infected with *B*-virus. The relatively tremendous blood concentrations of succinyl sulfathiazole which were produced repeatedly cannot be said to have caused definite pathological changes. Those differences which existed between the findings in the control and in the experimental animals are ascribed to changes of a compensatory character. Even the local damage at the sites of injection is not remarkable when it is considered that the 25 per cent solution used is extremely hypertonic.

**DISCUSSION.** The bacteriological data suggest that the monkey, under the dietary conditions of the experiment, is not an ideal animal for the demonstration of the chemotherapeutic activity of sulfonamides on the coliform organisms of the intestine. The unusually low coliform counts which were obtained in the feces of normal monkeys might suggest that succinyl sulfathiazole should reduce the number of these bacteria to 100 or less with even greater ease than in dogs. The cause of the lesser susceptibility of these organisms to succinyl sulfathiazole in the monkey is an intriguing question. Several possible factors may be involved; particularly attractive at the moment is the possibility of the presence of substances which inhibit the anti-coliform activity of the drug. Such hypothetical substances might be contributed by the non-coliform organisms which predominate among the intestinal flora, by the particular diet employed, or by the intestinal secretions of the monkey. The work of Poth and his collaborators (2, 3, 4) indicates that the factors which operate in the monkey appear to be of relatively little significance in the canine and human species.

The toxicological data show that succinyl sulfathiazole is absorbed to a very limited extent in mice, rats and monkeys; similar results were reported by Poth and co-workers (2, 3, 4) with dogs and human beings. Oral dosage in mice, with amounts up to 4.0 grams per kgm., caused no deaths and produced a blood level of only 2.6 mgm.% sulfathiazole and 4.2 mgm.% succinyl sulfathiazole. Addition of the drug (5%) to the diet of rats for a period of a month caused no toxic effects and failed to affect the growth rate. Prolonged administration to monkeys in amounts up to 5.0 grams per kgm. daily, in six divided doses, did not deleteriously influence the health of the animals and produced no evidence of pathological changes.

Parenteral administration of sodium succinyl sulfathiazole was resorted to in order to disclose any toxic effect of the drug which might result if absorption occurred under unpredictable circumstances. Injections of a neutral 25% solution of sodium succinyl sulfathiazole (1 gram per kgm. per day for ten days) in unilaterally nephrectomized monkeys caused damage at the sites of injection, but no other histopathological changes of consequence. The bone-marrow was more than normally active and increased vascularity of the glomerular tufts of the kidney was noted; this may have been due to the high concentration of the drug in the blood and glomerular filtrate. So prompt is the excretion of succinyl sulfathiazole following parenteral injection that concentrations as high as 12 grams per 100 cc. have been found in the urine. It is possible that succinyl sulfathiazole may have a sufficiently low renal threshold to be of value in the study of kidney function.

Succinyl sulfathiazole so readily forms soluble salts that precipitation in the urinary tract at times did not occur following parenteral administration, even with high concentrations, e.g. 10%; on the other hand, heavy crystallization has been noted with a concentration of 4%. The acidification of a dilute (0.2%) solution of sodium succinyl sulfathiazole in urine will result in precipitation of the drug. The occurrence of crystallization in urine is evidently dependent for the most part on the reaction; as the pH of the urine declines, the possibility of pre-

of these findings can be attributed to normal physiological responses of a compensatory nature. Four of the six animals, *including the control*, showed evidence of lymphoid hyperplasia, hyperemia of the adrenal medulla, tiny areas of necrosis in the liver, some desquamation of tubular epithelium of the residual kidney, and some pulmonary round cell infiltration. These changes may have been in part due to tuberculosis, although the animals had a negative tuberculin reaction at the beginning of the experiment. There is an interesting resemblance

TABLE 5

*Concentration of sulfonamides (in mgm. per 100 cc.) in the blood of unilaterally nephrectomized monkeys following the daily parenteral administration of sodium succinyl sulfathiazole (1 gram per kgm.)*

DAY	HOURS FOLLOWING INJECTION	I.V.*		I.V.		I.P.		I.M.		S.C.	
		Free†	Total†	Free	Total	Free	Total	Free	Total	Free	Total
1	0.2	3.5	152	4.1	170	—	74.5	—	136	—	65.1
	0.5	3.5	120	3.3	119	—	60.4	—	122	—	81.7
	1	2.7	104	2.9	98.0	—	44.7	—	93.3	—	91.0
	2	2.1	78.4	2.7	62.3	—	22.9	—	40.8	—	57.1
	4	2.3	32.3	2.7	22.3	—	8.2	—	12.5	—	10.4
	8	0.9	8.5	0.9	6.9	—	—	—	—	—	—
	24	0.5	6.3	0.9	7.5	—	8.2	—	7.8	—	7.2
	3	—‡	26.7	—	28.5	1.0	62.3	0.6	36.1	1.4	74.6
	24	—	15.1	—	—	—	—	—	6.0	—	9.4
7	4	—	39.8	—	40.8	—	—	—	—	—	—
8	4	—	—	—	—	—	52.1	—	38.0	—	55.2
10	0.2	4.5	167	4.3	138	—	—	—	—	—	—
	0.5	2.9	136	4.8	171	4.2	150	2.8	141	2.9	133
	1	2.4	102	3.0	125	2.9	115	2.7	128	2.8	138
	2	1.8	68.4	2.3	74.2	2.5	99.3	2.0	94.1	2.1	103
	4	1.2	25.4	1.5	25.4	1.7	53.0	1.1	38.9	1.4	49.9
	8	—	10.4	—	8.8	0.8	6.9	—	7.2	1.1	9.4
	24	—	4.7	—	3.5	—	8.8	—	8.8	—	6.3

\* The designation of each monkey in the table corresponds with that of table 4.

† See footnote.<sup>3</sup>

‡— = Very faint color (less than 0.5 mgm. per 100 cc.).

of some of the lesions to the pathological changes which Sabin and collaborators (11, 12) described in monkeys infected with *B*-virus. The relatively tremendous blood concentrations of succinyl sulfathiazole which were produced repeatedly cannot be said to have caused definite pathological changes. Those differences which existed between the findings in the control and in the experimental animals are ascribed to changes of a compensatory character. Even the local damage at the sites of injection is not remarkable when it is considered that the 25 per cent solution used is extremely hypertonic.

cipitation of succinyl sulfathiazole is greatly increased. So little drug is absorbed following *oral* administration, however, that no evidence of crystal formation or concretions was seen in the urine or urinary tract.

All information available at present indicates that it is unlikely that succinyl sulfathiazole will cause manifestations of serious toxicity following the oral administration of the drug.

The microscopic studies of tissues have been carried out with the aid and advice of Dr. Dale R. Coman, of the Department of Pathology, University of Pennsylvania School of Medicine. We are grateful for the valuable assistance rendered by our associates Miss Esther Terry, Miss Ethel Williams and Mr. Wilbur Benson.

#### SUMMARY

Succinyl sulfathiazole, 2-(*N*<sub>4</sub>-succinyl-sulfanilamido)-thiazole, a new sulfonamide of promise as an intestinal bacteriostatic agent, has been studied from the standpoint of acute and chronic toxicity.

Absorption of the drug from the gastro-intestinal tract has been shown to be very limited. No toxic effects were observed in *mice* following oral doses of 40 grams per kgm. Two hours after such doses blood concentrations of 2.6 mgm.% sulfathiazole and 4.2 mgm.% succinyl sulfathiazole were produced. *Rats* exhibited no depression of the growth rate and no other manifestations of toxicity when fed for 33 days on a chow containing 5% succinyl sulfathiazole. The mean blood concentrations found were 1.4 mgm.% sulfathiazole and 4.6 mgm.% succinyl sulfathiazole. *Monkeys* were given a solution of sodium succinyl sulfathiazole by stomach tube every 4 hours for 30 days in dosages up to 5.0 grams per kgm. per day. All animals gained weight and no toxic effects were seen; microscopic examinations of the tissues revealed no abnormalities. The average blood concentrations on the maximum dosage were 0.8 mgm.% sulfathiazole and 3.0 mgm.% succinyl sulfathiazole; calculations based on the maximum concentration found in the urine indicated that less than 4% of the ingested dosage (5 grams per kgm.) was excreted by the kidneys.

The effects of parenteral administration of succinyl sulfathiazole were studied to disclose any toxicity which might be caused by high blood and urine concentrations, should these occur under unpredictable circumstances following oral administration. By various parenteral routes one gram per kgm. (in the form of a neutral 25% solution of the sodium salt) was given to undilaterally nephrectomized *monkeys* daily for 10 days. The greater the local damage at the sites of the injections, the more significant was the decrease in hemoglobin concentration and erythrocyte count; these changes were not seen in all monkeys. Moderate activity of the bone-marrow was apparently compensatory to the decrease in blood constituents. Increased vascularity of the glomeruli of the kidney was ascribed to the high concentrations of drug in the blood and glomerular filtrate. In the blood, concentrations up to 170 mgm.% were reached (following intravenous injection); and in the urine a maximum concentration of 12 grams per 100 cc. was found (following intramuscular injection). Crystal formation in the

urine, which occurred irregularly, was apparently related primarily to urinary pH; no blockage of the urinary tract occurred and no crystals were found at autopsy.

Injected intraperitoneally in *mice* as an olive oil suspension, succinyl sulfathiazole had an approximate LD<sub>50</sub> of 5.7 grams per kgm. Blood concentrations two hours following the administration of the LD<sub>40</sub> dose of drug were 8.2 mgm.% sulfathiazole and 263 mgm.% succinyl sulfathiazole.

Injected intraperitoneally as an aqueous solution of the sodium salt, the LD<sub>50</sub> was found to be approximately 7.5 grams per kgm.; 7.0 grams per kgm. (the approximate LD<sub>40</sub>) produced a blood concentration of 9.7 mgm.% sulfathiazole and 775 mgm.% succinyl sulfathiazole, 30 minutes following drug administration.

All evidence available at present indicates that toxic reactions of consequence will not occur following the oral administration of succinyl sulfathiazole.

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# VISCERAL LESIONS ASSOCIATED WITH TRIBROMETHANOL ADMINISTERED RECTALLY<sup>1</sup>

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In the course of a series of experiments aimed primarily at a determination of the comparative antidotal efficiencies of picrotoxin and coramine in avertin narcosis (1), it was observed that two distinct types of death occurred from avertin intoxication. In the first instance there was acute death due to direct systemic poisoning where the dose was lethal and where the antidote when employed failed to protect. Twenty-eight out of a total of 89 animals exhibited this type of death. In the second instance, there occurred a delayed type of death following the administration of a sub-lethal dose of avertin or, in the case of a dose within the lethal range, when the antidote proved effective in protecting the animal against the acute systemic action of avertin. In this latter instance there occurred a recovery from the direct action of the narcotic and the animal regained consciousness, but without relapsing into narcosis or exhibiting any other sign of the deleterious effect of the avertin, death occurred between the first and seventh experimental day. Thirty-five of the 89 animals exhibited this type of delayed death. This report represents the results of a study into the mechanism causing the delayed type of death in animals subjected to avertin medication.

Rabbits were used throughout. Liquid avertin with amylen hydrate was administered per rectum either in single or in broken doses. One cc of the solution represented one gram of avertin crystals and 0.5 cc of the adjuvant amylen hydrate. The analeptics, picrotoxin and coramine, were injected intravenously.

Sixteen of the 35 animals included in our observations died during the day, and we were able to observe and record the symptoms associated with their deaths and also to remove immediately selected samples of tissues for histological study. In all the 35 instances, however, regardless of the time of death, an autopsy was done and visceral changes were studied grossly.

A constant phenomenon associated with these delayed deaths was acute pain on defecation. The animal would be apparently well when suddenly in an effort to defecate it would emit a piercing shriek and topple over in paroxysms of pain. This was soon followed by death. Without exception autopsy revealed inflammatory or ulcerative lesions in the colon, 3 to 4 inches above the anus. Thirteen cases (43%) of the series showed that the intestine had ruptured,

<sup>1</sup> We wish to express our thanks to Dr. W. S. Quinland, Professor and Head of the Department of Pathology at Meharry Medical College, and Dr. Robert S. Jason, Professor and Head of the Department of Pathology at Howard University Medical School, for their kindness in preparing and reading the slides.

throwing fecal material into the abdominal cavity. At the site of rupture the intestine usually showed signs of profuse granular corrosion. See table 1.

TABLE 1  
Delayed deaths

NO.	AVERTIN mgm/kgm	STIMULANT mgm/kgm	INITIAL RECOVERY TIME minutes	CONDITION FOLLOW- ING RECOVERY	DEATH AFTER RECOVERY
1	150		170	Good	On 5th day
2	250		270	Good	On 6th day
3	250		87	Very good	On 4th day
4	250	P 0.5 (1)	45	Very good	On 9th day
5	300		180	Good	On 4th day
6	300		300	Fair	On 4th day
7	300		180	Good	On 5th day
8*	300	C 685 (4)	245	Sick	On 4th day
9*	300	C 2250 (9)	480	Sick	In 48 hours
10*	300	C 416 (3)	300	Sick	In 12 hours
11*	300	C 315 (2)	300	Sick	In 12 hours
12*	300	C 700 (4)	244	Sick	In 12 hours
13	300	C 1805 (9)	367	Convulsed	Never awoke
14	300	C 385 (3)	145	Good	On 6th day
15	300	P 1.7 (1)	185	Very good	On 7th day
16	500		157	Very good	On 2nd day
17	500		148	Fair	On 1st day
18	500	C 1020 (8)			Never awoke
19	500	P 4.7 (3)	70	Very good	On 3rd day
20	500	P 3.8 (3)	95	Very good	On 4th day
21	500	P 2.7 (2)	47	Very good	On 7th day
22	500	P 3.5 (3)	84	Very good	On 5th day
23	750			Never awoke	On 3rd day
24	750			Never awoke	On 2nd day
25*	750	C 165 (1)	280	Sick	On 4th day
26*	750	C 555 (4)	1400	Sick	On 2nd day
27*	750	C 165 (1)	308	Sick	On 4th day
28	750	P 2 (1)	168	Good	On 3rd day
29	750	P 3 (1)	160	Very good	On 3rd day
30*	750	P 2.3 (1)	90	Convulsed	On 2nd day
31*	1000	C 435 (3)	Awoke next day	Sick	On 2nd day
32*	1000	P 2.7 (3)	Awoke next day	Sick	On 2nd day
33*	1000	P 4 (3)	Awoke next morning	Sick	On 2nd day
34	1000	P 2.8 (3)	Awoke next morning	Good	On 4th day
35*	1000	P 3.1 (3)	Awoke next morning	Sick	On 3rd day

Bracketed numbers under "Stimulant" indicate the number of injections. P = Picrotoxin, C = Coramine. Asterisks indicate perforations.

Histological studies for the purpose of determining the nature and extent of visceral lesions, if any, were made by Drs. W. S. Quinland and R. S. Jason, of the Meharry Medical College and Howard University School of Medicine, respectively. Although the tissues studied were not taken from the same animals, the general concurrence of these independent pathological findings makes it

unnecessary to report them separately. Following is a brief description of each group of samples:

**Brain.** Sections of cerebrum and cerebellum show demyelinization and fatty degeneration of the subarachnoid region, so that the neuroglia cells are more conspicuous. Several of the pyramidal cells of the cerebral cortex are swollen and are in a state of early granular degeneration; these appear more acid staining. The same applies to the large cells of the ganglionic layer of the cerebellum. Many of these cells also show complete destruction of the nucleus.

**Spleen. Normal.**

**Pancreas.** Some lysis; edema of its stroma and what appears to be fat necrosis, mild, but without inflammatory reaction.

**Liver.** Diffuse autolytic destruction of practically all the hepatic cells which now appear as shadows, with well defined cell walls that are somewhat exaggerated in staining reaction as a result of the persistent bile capillaries. The nucleolus is conspicuous in many of the nuclei. The cytoplasm is reduced to an attenuated area of acid staining amorphous material significant of massive edema that is further characterized by the generalized increase in size of the cells. These findings connote a primary cloudy swelling and subsequent autolysis such as may be seen in a profound toxemia. That the drug has spent most of its potency in the central portion of the lobules is attested by the gradual shading of incomplete destruction of the hepatic cells immediately surrounding the portal areas, where in this location, some persist as delicate fibrillary cords.

**Kidney.** Inflammatory lesions, resembling those found in the liver, which in some areas extend into the pyramids. The convoluted tubules are lined by cells which appear swollen and have granular cytoplasm. The picture is that of an early toxic nephrosis.

**Lungs. Essentially normal.**

**Heart.** Hematoxylin and Eosin reveal some inequality in the staining of many myocardial fibers. Fat stains show these to be granular, but it is with difficulty that droplets of fat are found indicative of very slight fatty degeneration.

**Colon.** The epithelium of the crypts shows mucoid degeneration and even disintegration. The mucosal stroma is edematous, somewhat engorged, shows small hemorrhages and is infiltrated slightly, but definitely by leucocytes, which are seen best next to the muscularis mucosa.

**Summary.** A severe catarrhal colitis is associated with degenerative changes in the heart, liver and kidneys, which is clearly fatty in the liver and indefinitely so in the heart and kidneys. There are questionable changes in the brain. The sinusoids of the spleen are dilated.

Solutions of avertin tend to decompose into dibromacetaldhyde and hydrobromic acid upon exposure to air, light or heat at 40°C or above. These substances are both very irritating to the rectal mucosa. As far back as 1931 Parsons (2) reported that cats, injected rectally with avertin solution previously decomposed by heat, developed acute hemorrhagic proctitis within a very short time. In the course of the discussion following his report Parsons stated that solutions of avertin made up with amylen hydrate are fairly stable. However, it is always routine procedure to test for break-down acid products by the simple use of Congo Red (2 drops of 1:1000 solution to 5 cc of the mixture). Nevertheless, a negative test is not always assurance against rectal irritation. In 1935 Shipway (3) listed colitis and intestinal obstruction among the contraindications for the use of avertin rectally. In the same year Barlow (4), discussing late death in rabbits anesthetized with avertin fluid administered by this route,

said: "Such late deaths were presumably due to local changes in the rectum and coecum as observed at autopsy, and ranged in degree from marked inflammation to necrosis with perforation and peritonitis." With these observations our gross findings (1) reported in 1940 are in substantial accord. In 1938, Beecher (5) cited two undesirable side actions to avertin administered rectally—transient diarrhea and rectal irritation. He listed these as factors contributing to the decline in its employment for surgical anesthesia. Amylene hydrate does not enhance the irritant action of avertin. It renders the solution more stable. In the course of the discussion of Parsons' report, referred to above, it was stated that Lundy administers avertin in 6% gum acacia with good results.

#### SUMMARY

1. Avertin administered rectally to rabbits in sub-lethal doses or in lethal doses which are effectively antidoted by picrotoxin so as not to cause acute death by systemic intoxication, produces a delayed death by rectal irritation, inflammation, ulceration and sometimes perforation.
2. This intestinal lesion is associated with analogous degenerative visceral changes chiefly in the heart, the kidney, the brain, and the liver.
3. The immediate cause of death is either perforation of the intestine followed by peritonitis or exruciating pain on defecation.

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# THE ACTION OF QUINIDINE ON THE COLD BLOODED HEART

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Studies on the action of quinidine on the heart made soon after the introduction of that drug into the therapeutics of heart disease indicated that it was primarily a depressant substance, acting uniformly on heart muscle fibers and the specialized tissues. Lowering of rhythmicity, raising of threshold, slowing of conduction in auricle and ventricle and lengthening of refractory period were emphasized by Lewis, Drury, Iliescu and Wedd (1). However, Love (2) working later in Lewis' laboratory concluded that for the tortoise ventricle poisoned by quinidine, the absolute refractory period was actually shortened when measured by an accurate method that largely excluded conduction. In 1932, Gold and Modell (3) reported that in unanesthetized dogs quinidine caused sinus acceleration, and impairment of A-V conduction was never seen. These authors attributed the results of earlier investigators to the effect of the anesthetics used rather than of quinidine itself. Results similar to those of Gold and Modell were reported by Flaum (4) who observed the action of quinidine on rabbits. Nevertheless, many current writers will stress the depressant action of quinidine, particularly its supposed lengthening of refractory period (5). On account of this lack of agreement it appeared desirable to make further studies of the subject.

The majority of observations herein reported were made on the heart of the turtle *Pseudomys elegans* using the whole heart either excised or *in situ* or strips of auricular or of ventricular muscle. In a few cases the whole heart of the frog *Rana pipiens* was used. The effects of quinidine on the rate of spontaneous beating, threshold for electrical stimuli, conduction rate, refractory period and duration of electrical systole have been studied. The method employed in studying electrical activity was essentially that introduced by Craib (6). Its theoretical aspects have been elaborated by Wilson, MacLeod and Barker (7). This method has already been used in this laboratory to study the relations of electrical and mechanical systole (8) and also in an investigation of the action of digoxin (9). Details of the method are given in those papers. When muscle strips were used they were either placed on a filter paper or fixed in a small well in a paraffin block and bathed with phosphate buffered Ringer's solution. The tissues were rhythmically stimulated by condenser discharges and electrograms from two regions were recorded simultaneously by means of amplifiers and piezo electric ink writers. At each of the regions selected for recording one electrode was placed against the tissue while the other was placed at a distance of 2 or 3 cm. in a position such that a line joining the electrodes was normal to the direction of the strip. The recording electrodes in contact with the strip terminated in cotton wicks. Strips were rhythmically stimulated through platinum electrodes placed transversely near one end. To record from the intact heart one active electrode was placed on the auricle and the other on the ventricle. In some of these experiments a single indifferent electrode placed at a distance was made common to both channels; in others, an indifferent electrode was provided for each active one. The method of determining the

refractory period will be described later in the text. The drug was applied to the excised tissues in concentrations from 1 to  $2 \times 10^{-4}$  in Ringer's solution. Concentrated solutions containing a few mgm. were given to the intact turtles.

Figure 1 illustrates the records obtained from strips. The lower tracing in each case is from a region proximal to the stimulated end and the upper tracing from a region more distal. The pens do not write one above the other but the shocks introduce simultaneous deflections like S in record D. The Q-T interval is measured from the middle stroke of the diphasic Q wave where it crosses the iso-electric line to the corresponding point of the T wave (9). Because the interval shock to Q in any case may be shortened by increasing the strength and consequently the spread of the driving shocks, conduction time was always measured from proximal to distal Q rather than from shock to Q in the tracing from a single region. It should be noted that Q-T as it is defined here may be quite constant as conduction is slowed while the corresponding interval as measured from the beginning of R to the end of T in the electrocardiogram is lengthened. The reason for this is that the latter quantity contains the conduction times for both excitation and recovery. It will be seen on comparing A and F of figure 1 that both the Q and T waves are longer in F, in keeping with slowed conduction. Consequently, electrical systole of a given region is apparently longer and of the whole strip is actually longer but the interval between depolarization and repolarization at a given region remains unaltered. For consistency in the present work Q-T intervals from the electrocardiograms of whole turtles or intact hearts were measured from a given phase of Q-R-S to a given phase of T—a method analogous to that used on strips.

**DRUG EFFECTS. Effect on the Q-T interval and the refractory period.** When the Q-T interval of rhythmically driven strips of either ventricular or auricular muscle was measured before and after the application of quinidine it was found that its value rarely showed significant change, and when it did slight shortening usually occurred. Examples of two such experiments are shown in figures 2 and 3. In a single instance, the Q-T interval of an auricular strip lengthened appreciably. Figure 1 shows some shortening in a ventricular strip.

It has already been shown that in this type of preparation when Q-T and refractory period are measured at the same region almost identical values are obtained (8). The procedure used to determine refractory period in these experiments was somewhat different from that previously described. Regular driving shocks were applied in the usual manner near one end of the strip. The test shock, however, was put in about 1 cm. lower down very close to the proximal recording electrode. It was assumed that the absolute refractory period at this point began with depolarization, i.e. with the Q wave. Measurements were made to determine only the end of the refractory period in relation to repolarization indicated by the T wave. If the refractory period at the region of the test electrode was over, an extra electrical response was recorded, both there and later at the distal recording electrode. If the refractory period had not ended either no new complex appeared or a small complex would be recorded at the proximal electrode only which represented a response from some recovered region between the driving and the proximal electrodes. The latter effect is possible because the strong test shocks may spread back toward the regularly driven end of the strip which is normally the first region to recover. This method has certain advantages over that used previously in which the test shocks were applied at the same place as the driving shocks, for if recording also is done at that region the complexes are distorted by the shocks, whereas if it is done at some distance,

the *Q-T* interval there may be different from that where the shocks are applied. With the present method, if the test shock makes recognition of the *T* wave diffi-

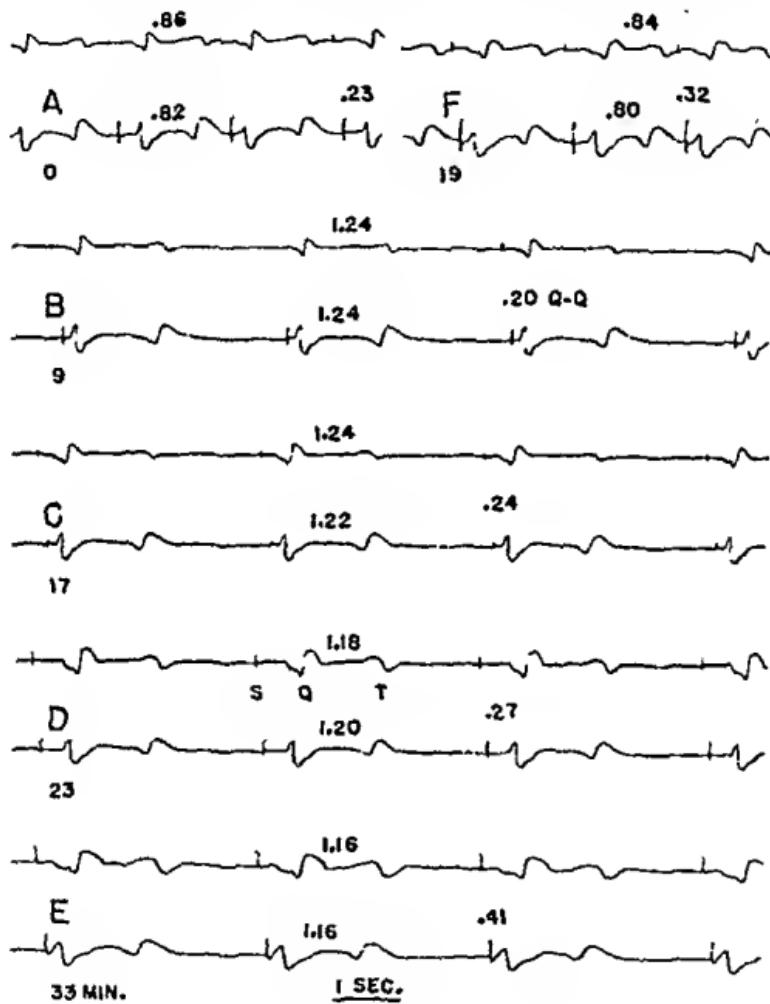


FIG. 1. TURTLE VENTRICULAR STRIP

cult it can be located by referring to the preceding regular complex. This is not entirely satisfactory but no determination of absolute refractory period is without error. The most serious difficulty with the present method occurs as a late drug effect in which conduction block is observed. This is discussed below.

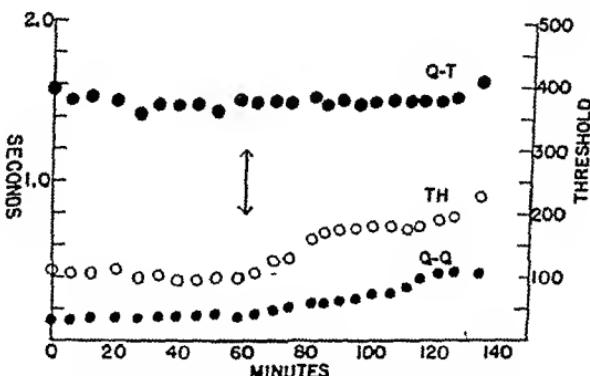


FIG. 2. TURTLE VENTRICULAR STRIP

The larger dots represent *Q-T* intervals, the smaller dots conduction times and the circles thresholds for electrical stimuli. At the arrow quinidine 1 to 5  $\times 10^{-3}$  was added to the irrigation fluid. The right hand ordinates represent thresholds only. The abscissae represent time from the beginning of the experiment.

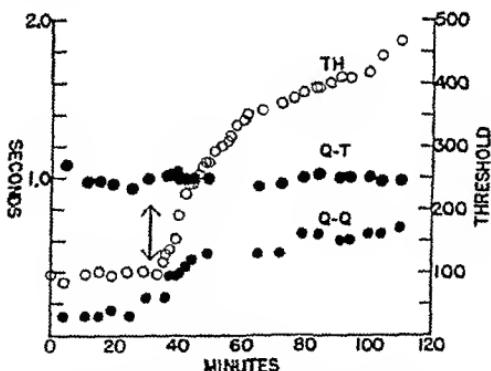


FIG. 3. TURTLE AURICULAR STRIP

The larger dots represent *Q-T* intervals, the smaller dots conduction times and the circles thresholds for electrical stimuli. At the arrow quinidine 1 to  $10^{-3}$  was added to the irrigation fluid. The right hand ordinates represent thresholds only. The abscissae represent the time from the beginning of the experiment.

Figure 4 represents data from one complete experiment. Measurements were made during 30 min. before and 50 min. after applying the drug. In the diagram the normal and drug results are to the left and right, respectively, of the arrow. In each determination the larger circle represents the time of writing of the *T* wave measured from its *Q*; the smaller circle represents the time of application of an extra shock which gave a response; the solid dot indicates an extra shock which failed to produce a response. Before applying the drug no shock

earlier than  $T$  by more than a small interval caused an extra beat, while no shock later than  $T$  failed to do so. After the drug has been on for some time the shocks had to be slightly later than  $T$  to be effective. This difference usually observed as a late drug effect is probably due to conduction block of the extra beat rather than to real lengthening of the refractory period. This is indicated by the observations that small un conducted responses are seen to shocks just following  $T$  and that the earliest conducted complexes are frequently weak as if due to response of only part of the strip. Only the shocks which produced conducted responses were plotted in figure 4. Block is to be expected at this time under the circumstances because added to the impairment of conduction brought about by the drug is the normal marked slowing associated with the early stages of the relative refractory period.

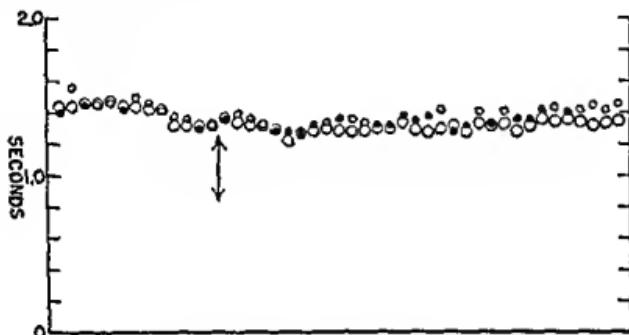


FIG. 4. TURTLE VENTRICULAR STRIP

The larger circles represent the times of occurrence of the  $T$  waves as measured from  $Q$ . The smaller circles show the times of application of test shocks which elicited extra systoles. The dots represent shocks which failed to elicit response. The abscissae have no significance as the measurements were made at irregular intervals for 30 minutes before adding quinidine 1 to 7500 at the arrow and for 50 minutes afterwards. The test shocks were kept at 5 times threshold strength as the threshold was raised by the drug.

It is concluded from experiments like those represented in figures 2, 3 and 4 that the  $Q-T$  interval is not altered by quinidine and that it is equal to the absolute refractory period after application of the drug as well as before. There is a slight apparent increase of the refractory period over the  $Q-T$  interval as a late drug effect but this is probably due to conduction block rather than to any real difference. Even if this effect should be brought about by therapeutic doses of the drug, which is doubtful, it is too small to demand serious consideration in explaining the action of quinidine. The effect of the drug on the excitation threshold and conduction are much more important. This will be considered now.

*Effect on threshold and conduction.* In figures 2 and 3 it will be seen that increase of the threshold for electrical stimuli begins as soon as the drug is applied. This invariable result is always accompanied by an increase of the conduction time. In the absence of changes of chronaxie it is to be expected that conduction effected by the action current of a tissue will be slowed in linear relation to the

reciprocal of increasing threshold as measured by externally applied shocks (10). This expectation is realized in nerve (11). Probably a sufficiently valid approximation is proportionality of conduction time to threshold. According to Moisset de Espanés (12) the chronaxie of toad heart is increased by quinidine. The dosage employed in his determinations was quite high but there is probably some change with small doses. If so the conduction time will increase more rapidly than expected from rise of threshold alone. In the experiments on which the present report is based approximate proportionality of conduction time to threshold was observed as an average result indicating that rise of threshold is the important factor in conduction change. Figure 5 represents qualitatively the threshold-conduction relation. It is to be expected on general grounds

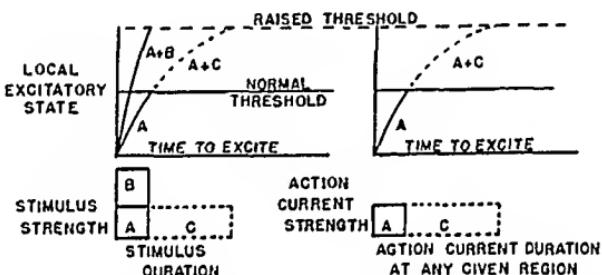


FIG. 5. THE RELATION OF CONDUCTION TO THRESHOLD

Illustrated on the left and right, respectively, is the relation of threshold of local excitatory state to external shocks and to action current. On the left the just adequate shock  $A$  represented by the rectangle raises the excitatory state  $A$  in the tissue to threshold value in a time just equal to its duration. If the threshold now rises to the level of the broken line the shock may be increased in strength to  $A + B$  just sufficiently to excite in the same time or it may be increased just sufficiently in duration to  $A + C$  leaving the strength unaltered. Thus a rise in threshold is indicated either by the necessity of increasing the strength or the duration of the externally applied stimulus. On the other hand, as represented on the right, when conduction is being effected by the action current of the tissue rise in threshold can be overcome only by increasing the duration of current flow at any given region because the strength of the current is determined by the membrane potential which is assumed not to have changed. Consequently, the action current is slowed by rise in threshold because the action current has to act longer to accomplish excitation. Pacemaker activity whether it is electrical or chemical. If the threshold is raised the pacemaker must act longer to accomplish excitation. Consequently, the rate of beating is slowed.

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*Q-R-S* lengthens because conduction is slowed. *Q-T* lengthens in response to the slowing of rate. The *P-R* interval in this case lengthens but this is not a constant result. In about equal numbers of experiments it lengthened, shortened and remained the same as the rate slowed. The basis for this finding is probably the observation that the *P-R* interval decreases to a minimal value and increases again as the rate of beating is decreased from a high value. Consequently, slowing any given preparation may either lengthen or shorten *P-R*. The drug might be expected to exert two actions, one a direct lengthening of the *P-R* by slowing conduction and the other a change in either direction through rate slowing. That the drug does lengthen *P-R* as a direct action appears, however, to be very doubtful.

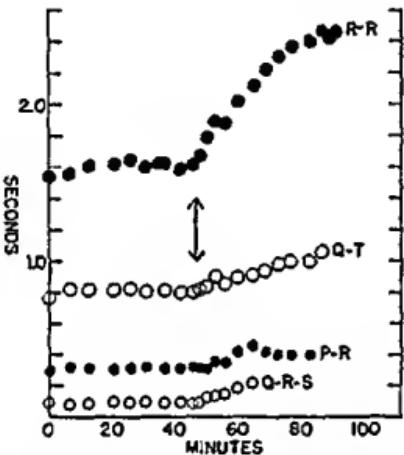


FIG. 6. TURTLE HEART *IN SITU* BEATING SIMULTANEOUSLY

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*The direct effect on the P-R interval.* Excised whole turtle hearts in Ringer's solution and a few *in situ* were driven at a constant rate, 18 per minute, for periods before and after the addition of quinidine to determine the variation of *P-R* in the absence of rate change. The stimuli were applied to the right auricle and measurements were made of the duration of the *P-R* interval and of the iso-electric period between *P* and *Q* so as to avoid in the latter case, including any part of auricular or ventricular conduction in the results. Unexpectedly, no important alteration of the iso-electric interval ascribable to drug action was found even when ventricular conduction had become so slow the *Q-R-S* was extended several fold and the beat was no longer coordinated. The duration of *P* was increased but it was never a large enough fraction of *P-R* to lengthen that period significantly. The experiments were not entirely satisfactory because *P-R* usually tended slowly to lengthen in Ringer's solution alone. In no case

following the drug, however, did *P-R* lengthen more than a few percent from any cause. This result makes it seem doubtful that *A-V* conduction in the turtle is mediated by unspecialized muscle fibres as a simple extension of auricular conduction.

*The effect of rate on Q-T before and after the drug.* To answer the question as to whether the variation of *Q-T* with rate as well as its value at a given rate is unaltered by quinidine a number of ventricular strips were driven at twice the usual rate of 18 per minute for periods before and after the drug. The results are illustrated by records *A* and *F* of figure 1. In *F* after the drug, *Q-Q* is much longer showing that drug action has taken place on conduction but *Q-T* is unchanged. It is indicated by these and similar results that the variation of *Q-T* with rate is not altered by the drug. It will be observed in figure 1 that conduction in *F* is slower than in *C* and *D*. Increase of rate under the drug in this case slowed conduction but this was not a constant finding and the conditions for its appearance were not discovered.

**COMMENT.** It appears from these experiments that the action of quinidine on cardiac muscle is relatively simple and may be explained for the most part by the raised threshold for stimulation which it causes. This rise of threshold in turn slows muscle conduction. Because of the anti-vagal action of the drug the rate of beating of the normal cardiac rhythm of the intact mammal may be increased. However, rise of threshold and the anti-vagal action work together to slow conduction, at least in the auricle. Consequently, the drug may be expected to have its greatest therapeutic use in those cases in which slowing of conduction will be of advantage. The principal examples in which that condition obtains are the re-entrant tachycardias, fibrillation and flutter, for in these slowing of conduction slows the rate of the abnormal pacemaker and affords thereby greater opportunity for the normal pacemaker to resume control. Such opportunity is somewhat lessened by an increase of refractory period of the muscle consequent to decreased rate of beating and the anti-vagal effect on auricular muscle, but this may be offset by heightened rhythmicity at the *S-A* node. Similarly, the drug will be indicated in ventricular tachycardia, which is now believed to be a re-entrant mechanism and is known to be slowed by quinidine. In this case the anti-vagal action probably does not contribute to the slowing of conduction within the muscle but it does facilitate *A-V* nodal conduction, and since the latter does not appear to be slowed by direct action of the drug impulses from the increased rhythm of the normal pacemaker are assured conduction to the ventricle.

The use of the drug appears to be contra-indicated in either sinus or nodal tachycardia because in these the anti-vagal action will increase the rhythm of the pacemaker while slowing of muscle conduction may be harmful by producing a less co-ordinated ventricular beat.

#### SUMMARY

Studies made on driven strips of turtle auricle and ventricle have shown that quinidine does not alter significantly the absolute refractory period. The

refractory period and the *Q-T* interval have again been found to be practically equal, and this holds both normally and under quinidine action. The threshold for electrical stimuli is raised promptly and in consequence conduction in muscle is slowed, in about the same proportion. The drug slows the rate of beating of the heart of the pithed turtle and of the isolated frog heart. The *P-R* interval of the rhythmically driven turtle heart, *in situ* or isolated, is not appreciably lengthened at a time when conduction in auricle and ventricle is markedly slowed. Variation of *Q-T* interval with rate of beating is the same after the drug as before. It is concluded that the principal effects of the drug can be ascribed to rise of threshold and its consequences. Therapeutic use of the drug is indicated primarily in the re-entrant tachycardias, for in them slowing of conduction within the muscle may permit the normal pacemaker to re-establish control.

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# A STUDY OF THE SYNTHESIS OF HIPPURIC ACID IN THE RAT AFTER LIVER INJURY FROM CARBON TETRACHLORIDE<sup>1</sup>

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There have been comparatively few studies of the effect of liver injury by toxic agents upon liver function. The effect of hepatic damage upon hippuric acid synthesis from administered benzoate has been especially neglected, although the Quick hippuric acid test (1) has been used as a clinical test of liver function since 1933. Carbon tetrachloride produces anatomical hepatic injury, and associated with the histological changes certain evidences of functional disturbance have been found through liver function tests. In dogs, dye retention and reduced galactose tolerance have been described (2, 3), while in rats a marked drop in the plasma prothrombin level occurs after carbon tetrachloride poisoning (4). Adlersberg and Minibek (5) studied the urinary excretion of hippuric acid in rabbits after carbon tetrachloride and phosphorus injections. Urine was collected for 4 hours after benzoate administration and the hippuric acid was determined by ether extraction and a formol amino titration. They found a decrease in hippuric acid excretion on the second or third day after daily injections of carbon tetrachloride were begun.

On the contrary, Bryan (6) failed to find definite evidence of diminished hippuric acid excretion in dogs after carbon tetrachloride, and Mann and Bollman (7) have stated that they have been unable to demonstrate any reduction in the rate of conjugation of benzoic acid in completely hepatectomized dogs. In these experiments total combined benzoic acid and not hippuric acid was determined.

The hippuric acid synthesis following certain other hepatotoxic substances has also been studied. Lackner, Levinson, and Morse (8) reported that hippuric acid synthesis was diminished in dogs after hydrazine poisoning, while Delprat and Whipple (9) obtained similar results in dogs after chloroform anesthesia. As has been shown (10), these findings are invalidated because in both cases the Folin-Flanders method for hippuric acid was used, and this determines total combined benzoic acid, not hippuric acid. Quick and Cooper (10) showed by Quick's method that chloroform only slightly decreased the synthesis of hippuric acid in dogs. Tulane, Christman, and Lewis (11) found in rabbits treated with hydrazine a significant decrease in the hippuric acid output six hours after sodium benzoate administration. Griffith's method (12) was employed which estimates actual hippuric acid. Kanzaki (13), from a study of the blood hippuric acid after phosphorus and chloroform poisoning,

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concluded that the liver was concerned with hippuric acid synthesis in the rabbit.

It was thought that more information could be obtained by studying hippuric acid synthesis in another species, the rat, in the presence of a normal liver and a liver damaged by carbon tetrachloride.

**METHODS.** For the test of hippuric acid synthesis, adult fasting rats were given by stomach tube 660 mgm. of benzoic acid per kilo as sodium benzoate in a 1.56% aqueous solution. The volume of the solution administered was 5% of the weight of the rat. The large water intake was employed to insure adequate urinary volume. Urine was collected for an eight hour period because of the slow rate of synthesis of hippuric acid in the rat, as is shown below. After the first 5 hours, a second diuresis was induced by administering water in the same volume as before. The determination of hippuric acid in the urine was performed according to the ether extraction method of Quick (1) which consists of the continuous ether extraction of a urine sample, HCl hydrolysis of the extracted hippuric acid, and a formol titration of the glycine liberated. Northrop's formol titration (14) was

### HIPPURIC ACID EXCRETION

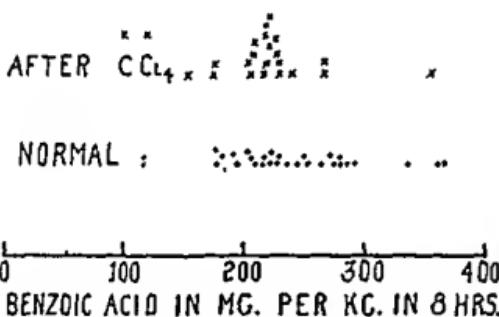


FIG. 1. HIPPURIC ACID EXCRETION (CALCULATED AS BENZOIC ACID) IN RATS DURING EIGHT HOURS FOLLOWING THE ADMINISTRATION OF SODIUM BENZOATE (660 MGm./KGM. OF BENZOIC ACID)

substituted for that described by Quick. The glycine found was calculated as benzoic acid. Urine samples were either extracted the same day or preserved in the ice box overnight under toluene.

Carbon tetrachloride in a dose of 1 cc. per kilo was injected subcutaneously in a 50% olive oil solution three times a week for periods up to 4 weeks. In a few acute experiments it was not diluted with oil but injected as such in a dose of 10 cc. per kilo. The livers of the rats were preserved in formalin and in Maximow-osmic fluid and studied histologically in order to correlate pathological and chemical findings. The diet of the animals consisted of Wayne Dog Chow.

**RESULTS.** The chemical procedures were checked with glycine and recrystallized hippuric acid. The titration error was found to be within 1% and the over-all error including urine extraction was within 10%. The fasting excretion of hippuric acid by normal rats in an 8 hour period without administered benzoate was found not to be significant. Values (calculated as benzoic acid) between 3 and 26 mgm./kgm. were found. After oral sodium benzoate the

rate of hippuric acid excretion in the rat was found to be lower than in the rabbit (12). This relationship agrees with *in vitro* studies (15). In a preliminary experiment, eight hours after sodium benzoate was given, 280 mgm./kgm. of benzoic acid were found, representing 42% of the benzoic acid administered, while in the next eighteen hours 81 mgm./kgm., or 12%, were found. The total for 24 hours was 361 mgm./kgm., representing a synthesis and excretion

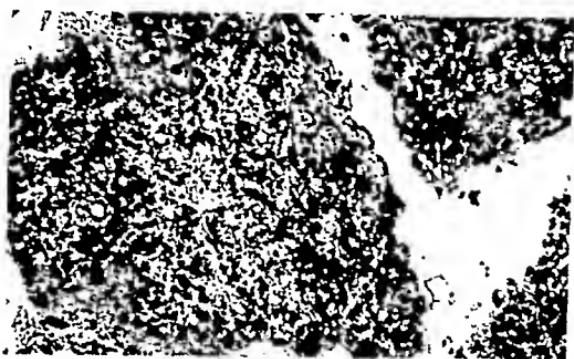


FIG. 2. MAXIMOW-OSMIC PREPARATION OF THE LIVER OF RAT NO. 3 SHOWING 5+ FATTY DEGENERATION FOLLOWING  $\text{CCl}_4$

TABLE I

*Hippuric acid excretion in rat no. 1 before and after carbon tetrachloride injections*  
Hippuric acid expressed as benzoic acid

DATE	WEIGHT	BENZOIC ACID GIVEN	BENZOIC ACID RECOVERED IN 8 HOURS	SYNTHESIS	BENZOIC ACID RECOVERED
	grams	mgm.	mgm.	per cent	mgm./kgm.
March 3	311	211	71.9	34.1	230
March 25.	297	198	109.9	55.5	367
April 18	322	211	66.2	31.3	207
April 30..	CCl <sub>4</sub> begun; 1 cc. 3 times a week				
May 7	328	211	68.1	32.2	206
May 15	300	198	79.2	40.0	264
May 19..	299	198	70.7	35.6	236
May 23	278	185	61.5	33.3	221
May 29	275	185	57.3	31.0	208

of 54%. The eight hour excretion value in normal rats varied fairly extensively, but in general fell between 175 and 275 mgm./kgm.

The result of carbon tetrachloride intoxication on the hippuric acid excretion is seen in figure 1. These values represent repeated experiments on 12 rats before and at various times after carbon tetrachloride was begun, ranging from 19 hours to 4 weeks after the first dose. It will be seen that the great majority of the results were within the normal range. Of the low values seen, one (120 mgm./kgm.) occurred in rat No. 6, 7 days after carbon tetrachloride was begun,

but it was followed by normal values of 176 and 226, 6 and 13 days thereafter. The liver damage in this rat was graded as 2+ on histological examination. The hippuric acid excretion in rat No. 2 was found to be 218, 207, and 228 mgm./kgm. (benzoic acid) on the 7th, 14th, and 17th days respectively after carbon tetrachloride injections were begun, but fell to 155 on the 21st and to 101 on the 26th day. The last figure was unreliable because of oliguria. The liver of this animal showed a 5+ (maximum) injury histologically. The data on this animal are suggestive of functional liver deficiency, but the results of tests on other animals similarly treated do not substantiate this conclusion. The remaining 6 rats maintained a normal hippuric acid excretion throughout the course of carbon tetrachloride treatment. One rat (no. 4) excreted 203 mgm./kgm. of benzoic acid even though it died at the end of the test. The liver of this rat showed a 5+ injury. It is notable that even in a moribund state and with a liver severely damaged from an anatomical standpoint, the animal was able to synthesize hippuric acid at a normal rate. The livers of all the animals showed damage to some extent (fig. 2). A protocol of a typical series of experiments on one animal is shown in table 1.

**DISCUSSION.** Obviously, the rate of synthesis of hippuric acid in rats poisoned with carbon tetrachloride is the same as in normal rats, in spite of the anatomical evidence of liver injury. These findings are directly at variance with those of Adlersberg and Minibek (5) in the case of the rabbit. The explanation of this discrepancy may thus be in a species difference which may apply (a) to the site of hippuric acid synthesis, or (b) to the degree of functional liver damage produced by carbon tetrachloride. In support of (a) it will be recalled that in the rabbit a decreased rate of hippuric acid synthesis occurs after chloroform, phosphorus (13) and hydrazine poisoning (11), and the synthesis in the last case is not increased by feeding glycine. In the dog total conjugated benzoic acid values remain unchanged after hepatectomy (6). Borsook and Dubnoff (15) have shown that the synthesis of hippuric acid occurs *in vitro* with dog kidney slices but not with dog liver slices; while in the guinea pig, rabbit, and rat it occurs with both liver and kidney slices. Apparently the older observations of Bunge and Schmiddeberg (16), that the kidney is essential for hippuric acid synthesis in the dog have been well substantiated. Clinical experiences have definitely associated the synthesis with the liver in man. In the case of the rabbit, it would seem that the liver is of great importance in this synthesis; while our findings indicate the relative unimportance of the liver of the rat in hippuric acid synthesis.

From the point of view (b) of a species difference in the degree of functional liver damage produced by carbon tetrachloride, Quick (1) has assumed that impaired hippuric acid excretion indicates deficient glycine formation in the liver. Our results would then indicate either an extrahepatic production of glycine in rats or a remarkable ability of the hepatic cells to produce glycine in spite of injury. Wells (17) has emphasized the ability of injured hepatic cells to function normally, and has pointed out that toxic fatty metamorphosis may fail to be associated with marked impairment of function. Thus, from this

point of view, our findings could be interpreted as showing that the rate of glycine production by the rat liver is normal even when the liver is severely damaged anatomically by carbon tetrachloride. At present it cannot be stated which point of view is correct.

#### CONCLUSIONS

1. Hippuric acid excretion following benzoate administration in the rat takes place at a slower rate than has been found by Griffith for the rabbit.
2. After acute or chronic hepatic injury produced by carbon tetrachloride, rats are able to synthesize and excrete hippuric acid from administered benzoate at a normal rate in an eight hour period.
3. Species differences in this detoxication mechanism must be taken into consideration in interpreting results.

We wish to thank Mr. Phillip Hitchcock for constructing the extractor used in these experiments and Dr. William H. Summerson of Cornell Medical College who criticized the manuscript.

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# STUDIES ON SYNTHETIC CURARE-LIKE COMPOUNDS

## I. ACTION OF SOME QUININE AND OTHER QUATERNARY AMMONIUM DERIVATIVES<sup>1</sup>

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The hazards accompanying metrazol therapy of schizophrenia were recognized by Stalker (1) and others shortly after this method of treatment was introduced. Attempts to prevent traumatic fractures by modifying the tonic muscular contractions with curare were first tried by Bennett (2) with some success, but the difficulty in obtaining a potent and reliable curare has always been an important factor in its use. Other agents have been suggested, and among these are salts of two naturally occurring alkaloids, beta-erythroidine hydrochloride and dihydro-beta-erythroidine hydrobromide, first isolated by Folkers and Major (3). Rosen and associates (4) have shown that beta-erythroidine can modify the metrazol seizures in dogs. Recently Williams (5) and Rosen and Borenstein (6) reported on a series of clinical trials of this substance, during which they encountered toxic manifestations. Synthetic curare-like agents have been tried also. Quinine methochloride dihydrate is one of these and has been investigated by Harvey (7). The drug possesses a marked curare-like action but Bennett (8) after clinical trial felt that the quinine derivative was not as satisfactory as curare. Prolonged apnea and a significant fall in blood pressure appeared to be the principal disadvantages.

The present paper describes 34 synthetic compounds belonging to the curare-like group of agents. A majority of these substances has been prepared before as chemical entities, but many of them have not been considered previously as possible curare substitutes. The following study was undertaken in the hope of establishing more definite data on the curare-like responses. For the purpose of comparison quinine methochloride dihydrate and the two erythrina alkaloids are included. To conserve space only pertinent references are cited.

**METHODS.** Each of the substances was administered to frogs and if it proved effective, further tests were carried out on rabbits and pigeons and occasionally on cats. Those which showed a curariform paralysis and no muscular tremors in frogs with doses less than 100 mgm. were examined for their respiratory and circulatory actions, using the modified method of Thomas and Franke (9, 10). With this arrangement it is possible to determine the functional state of the central respiratory mechanism by recording the rhythmic contractions of an excised leaf of the diaphragm in response to impulses mediated through the intact phrenic nerve. Any central stimulation or depression is indicated by an increase or decrease of the rate and amplitude of the contractions. Peripheral or curare-like paralysis is shown by cessation of activity of the intact leaf. Blood pressure was recorded from a carotid artery in the usual manner. The extent of peripheral muscular

<sup>1</sup> This research was supported by a grant from the Parke Davis Company.

TABLE 1  
*Comparison of curare-like effect of thirty-seven quaternary ammonium compounds*

DRUG	TOTAL NUM- BER OF OBSER- VATIONS	APPROXIMATE CURARIZING DOSE, MCM./KG.			REMARKS
		Frog	Pigeon	Rabbit	
Cr-1 Quinine methochloride dihydrate...	27	40	10	7.5	Active
Cr-2 2-Methylbenzoxazole benzobromide.	5	300			Ineffective
Cr-3 Trimethylene-bis-isothiourea hydrobromide.....	10	1000			Ineffective
Cr-4 Beta-hydroxyethoxethyltrimethyl ammonium chloride.....	13	30	2.5	5	Muscular tremors
Cr-5 Quinine ethochloride dihydrate....	14	30	10	5	Active
Cr-6 Dimethylaminoacetamide methochloride.....	10	300	20	20	Slight action
Cr-7 N-Methylephedrine methiodide....	19	30	50	15	Active
Cr-8 Phenacyltrimethyl ammonium chloride.....	10	50	10	5	Muscular tremors
*Cr-9 3,4-Dihydroxybenzoylmethyltrimethyl ammonium chloride.....	8	30	10	10	Muscular tremors
Cr-10 Alpha-picoline-beta-naphthoylmethobromide.....	12	300	100	20	Ineffective
Cr-12 Alpha-picoline methiodide.....	9	300	100		Ineffective
Cr-14 Phenethyltrimethyl ammonium bromide.....	10	15	5	5	Muscular tremors
Cr-15 Trimethylene di-(trimethyl) ammonium chloride.....	9	300	100		Muscular tremors
Cr-16 Cetyl trimethylammonium bromide.	14	200	15	20	Muscular tremors
Cr-17 Quinoline methiodide.....	9	300	100		Slight action
Cr-18 Benzyltrimethyl ammonium bromide.....	8	40	10	4.5	Muscular tremors
Cr-19 Cetylpyridinium bromide.....	6	300	100		Slight action
Cr-20 Para-nitrobenzyltrimethyl ammonium bromide .....	7	100	25		Muscular tremors
Cr-21 Trimethylacetoximino ammonium chloride.....	5	300			Ineffective
*Cr-22 2-aminopyridine phenacyl bromide..	6	300			Muscular tremors
*Cr-23 Trimethyl-alpha-acetaminopyridyl ammonium chloride.....	6	200			Slight effect
Cr-25 Trimethylaminoethyl acetate chloride.....	5	300			Ineffective
Cr-26 Phenylisopropyltrimethylammonium iodide.....	8	30	5	7.5	Muscular tremors
Cr-28 Nicotinic acid methiodide.....	5	300			Ineffective
Cr-29 Brucine methiodide.....	12	70	15	15	Active
*Cr-30 Brueinc methobromide.....	10	60	15	15	Active
Cr-31 Quinine methobromide.....	12	50	10	5	Active
Cr-32 Quinine di-methiodide.....	6	150			Active
Cr-34 Beta-ethoxyethylmethylmorpholinium iodide.....	5	300			Ineffective
Cr-35 Beta-hydroxyethylmethylmorpholinium iodide.....	5	300			Ineffective

TABLE I—Concluded

DRUG	TOTAL NUM- BER OF OBSER- VATIONS	APPROXIMATE CURARIZING DOSE, MG/M/KG			REMARKS
		Frog	Pigeon	Rabbit	
*Cr-36 Beta-hydroxyethylbenzylmorpholinium iodide.....	5	300			Ineffective
*Cr-37 Phenylmethylmorpholinium iodide	5	300			Slight action
Cr-38 Quinine methiodide.....	7	60			Active
Cr-39 Trimethylphenylammonium iodide	5	150			Active
Cr-40 Trimethyl-para-tolylammonium iodide .....	7	80			Active
Beta-erythroidine HCl.....	15	5	5	5	Active
Dihydro-beta-erythroidine HBr....	13	2	2.5	1	Active

With the exception of beta-erythroidine hydrochloride and dihydro-beta-erythroidine hydrobromide, all of these compounds were supplied by the Research Division of Parke-Davis & Company, under the direction of Dr. Oliver Kamm. The alkaloids were supplied by Dr. D. F. Robertson of Merck & Company.

\* These compounds on the list are new, having been made for the first time in connection with this work.

paralysis was determined by electrical stimulation of a sciatic nerve. All drugs were injected into a lymph sac in frogs and intravenously in the other species. The doses are given as milligrams per kilogram.

**RESULTS.** A list of the compounds and a summary of their effectiveness are given in table 1. From this preliminary study the most active compounds appeared to be, besides quinine methochloride (Cr-1) and the erythrina alkaloids, Cr-5, Cr-7, Cr-29, Cr-30, Cr-31, Cr-38, Cr-39 and Cr-40. Further analysis of their actions on the respiratory and circulatory systems served to bring out certain undesirable side actions and eliminated all but Cr-5 from possible future consideration. Cr-7 produced only transitory peripheral paralysis in doses up to 40 mgm. Higher doses were not tried because the active pressor effect of this compound seemed to preclude further study. Cr-29 and Cr-30, although effective in doses of 10 to 20 mgm., appeared to increase the excitability of the spinal cord. Tremors and moderate tetanic convulsions were observed which undoubtedly were considerably conditioned by the curare-like action of these substances. No significant difference was noted in the actions of the three methohalogen derivatives of quinine, Cr-1, Cr-31 and Cr-38. Cr-39 and Cr-40 showed a marked muscarine-like effect. Table 2 is an evaluation of the actions of Cr-5, quinine ethochloride dihydrate, quinine methochloride dihydrate and the erythrina alkaloids.

The smallest dose which produced a distinct modification of the contractions of the intact diaphragm was found to be about 5 mgm. for Cr-1, Cr-5, and beta-erythroidine. However, for prolonged effects 17.5 mgm., 12.5 mgm., and about 7.5 mgm. respectively, were required. Dihydro-beta-erythroidine was the most potent of the group, 0.5 to 1.0 mgm. producing complete diaphragmatic paral-

ysis. With respect to the central respiratory mechanism none of these agents could be classed as depressants unless 4 to 10 times the minimal paralytic dose was administered. Only a moderate fall in blood pressure was observed with any of these substances. This fall usually accompanied injection of the drug and recovery occurred within 3 to 5 minutes. Cr-1 produced the greatest and

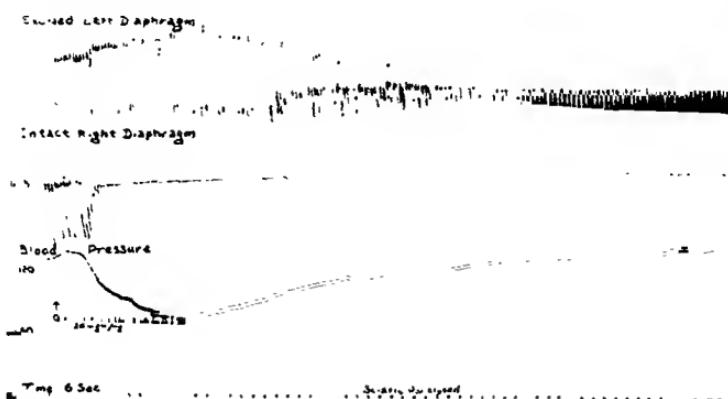


FIG. 1. TYPICAL EFFECTS OF A QUININE DERIVATIVE (QUININE METHOBROMIDE) ON CONTRACTIONS OF INTACT AND EXCISED SEGMENTS OF THE DIAPHRAGM IN A DOG

About two times the paralytic dose was injected intravenously. Immediate paralysis of the intact segment occurs. The isolated leaf maintains rhythmic contractions indicating no central respiratory depression. A moderate transitory fall of blood pressure may also be observed.

TABLE 2

*Summary of effects of curare-like agents on respiratory mechanism and blood pressure in dogs*

COMPOUND	DOSEAGE RANGE mgm./kgm.	NUMBER OF EXPERIMENTS	EFFECT ON			
			Blood pressure per cent	Central respiratory mechanism	Peripheral respiratory mechanism	Sciatic
*Quinine ethoehloride dihydrate	5-25	7	-33	None	Complete paralysis	Complete paralysis
Quinine methoehloride dihydrate	5-15	8	-41	None	Complete paralysis	Partial to complete
Beta-erythroidine-HCl	0.5-15	15	-14	None to slight	Partial to complete	None to complete
Dihydro-beta-erythroidine-HBr.	0.5-20	6	-20	Slightly depressed	Complete paralysis	Complete paralysis

\* First prepared by Streicher, Ann., 91: 167, 1854.

beta-erythroidine the least fall in blood pressure. Figure 1 shows a typical response of one of the quinine derivatives, quinine methobromide.

#### SUMMARY AND CONCLUSIONS

1. Thirty-four synthetic agents belonging to the curare-like group of compounds were studied for their peripheral paralyzant action.

2. Compounds possessing three methyl groups presented muscarine-like actions characteristic of tri-methyl ammonium compounds. The curare action was not markedly changed by alteration in chemical structure and such agents seem to hold little promise as curare substitutes for therapeutic application.

3. Beta-erythroidine hydrochlorido and dihydro-beta-erythroidine hydrobromide are effective neuromuscular paralyzants, the latter having about five times the potency of the former. A central respiratory depression was produced by these substances, the amounts required, however, being several times the fatal paralytic dose.

4. The methyl ester of quinine has about four times the potency of the dimethyl ester. On the other hand, quinine ethochloride dihydrate has an activity approaching twice that of the monomethyl derivative. Duration of action appears to be somewhat shorter for the ethyl ester which may be an advantage in its use in shock therapy.

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## STUDIES ON SYNTHETIC CURARE-LIKE COMPOUNDS

### II. SOME QUANTITATIVE COMPARISONS OF QUININE ETHOCHLORIDE DIHYDRATE, QUININE METHOCHLORIDE DIHYDRATE, BETA-ERYTHROIDINE HYDROCHLORIDE AND DIHYDRO-BETA-ERYTHROIDINE HYDROBROMIDE<sup>1</sup>

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In a previous communication (1) a series of curare-like compounds were described. Quinine ethochloride dihydrate and dihydro-beta-erythroidine hydrobromide gave definite promise of therapeutic usefulness as neuromuscular paralyzants. Since unfavorable clinical results have been reported on quinine methochloride (2, 3) and beta-erythroidine hydrochloride (4) when used to modify metrazol seizures in shock therapy, it seemed desirable to make a quantitative comparison of these curare-like agents with quinine ethochloride dihydrate and dihydro-beta-erythroidine hydrobromide.

**Toxicity.** This was determined in dogs and rats. The drugs were administered as 1% solutions intravenously in dogs and intravenously and intraperitoneally in rats. The effects of toxic doses of these substances were characteristically those of a paralytic dose of curare. Death in every instance was due to peripheral respiratory paralysis, as diaphragmatic breathing persisted to the last and the heart continued beating 3 to 8 minutes after all attempts at respiration had ceased. The erythrina alkaloids presented characteristic responses not observed with the quinine derivatives. These consisted of a marked slowing of the heart especially in dogs, from a control of 120-140 to 24-40 beats per minute. Salivation was also noted in all animals. In surviving dogs severe gastrointestinal distress with retching, vomiting, repeated attempts at defecation and urination almost always occurred. The animals assumed a crouched position giving every evidence of acute abdominal pain, and were depressed for an hour or more. A summary of the toxicity of the four compounds is given in table 1.

It is seen that fatal doses when administered intravenously were considerably lower for the quinine derivatives in white rats than in dogs. For instance, the surely fatal (100 per cent mortality) dose for white rats was 6 mgm. per kilo for quinine ethochloride dihydrate and 10 mgm. per kilo for quinine methochloride dihydrate, while that for normal dogs was approximately twice as large, or 15 mgm. and 20 mgm. per kilo respectively. Strangely enough the opposite obtains with the erythrina alkaloids. Dihydro-beta-erythroidine is decidedly less toxic in rats than in dogs. The dose for 100 per cent mortality of white rats was 15 mgm. per kilo as compared with 1 mgm. per kilo in dogs. Similarly for beta-

<sup>1</sup> This research was supported by a grant from the Parke-Davis Company.

erythroidine, 50 mgm. per kilo in rats killed 8 of 9 animals, while 10 mgm. per kilo proved fatal in each of 3 dogs. No comparison of intraperitoneal toxicity was made in dogs and rats. However, in comparing intravenous and intraperitoneal doses in rats it may be seen that a wider margin of dosage exists between the amounts necessary for a fatal outcome for the quinine derivatives

TABLE I  
Toxicity

SUBSTANCE	INTRAVENOUS IN DOGS			INTRAVENOUS IN RATS			INTRAPERITONEAL IN RATS		
	Dose mgm. per kgm.	Number of animals	Percent mortality	Dose mgm. per kgm.	Number of animals	Percent mortality	Dose mgm. per kgm.	Number of animals	Percent mortality
Quinine ethochloride dihydrate	5.0	1	0	5.0	5	40	25.0	6	16
	7.0	1	0	6.0	5	100	37.5	5	60
	8.5	3	33	7.0	5	100	40.0	5	60
	9.0	3	0	7.5	5	100	50.0	5	100
	10.0	3	33						
	11.0	2	0						
	12.5	3	0						
	15.0	2	100						
Quinine methochloride dihydrate	5.0	2	0	5.0	5	60	50.0	3	0
	10.0	3	0	7.5	5	80	60.0	5	0
	15.0	3	33	10.0	5	100	70.0	5	20
	17.5	3	66				75.0	7	71
	20.0	3	100				100.0	3	100
							125.0	3	100
							150.0	3	66
Dihydro-beta-erythroidine hydrobromide	0.75	3	0	0.5	3	0	10.0	3	0
	1.0	3	100	5.0	5	20	25.0	5	0
	2.0	3	100	7.5	11	45	30.0	8	25
				10.0	15	66	35.0	5	50
				12.5	10	60	37.5	3	100
				15.0	5	100	50.0	3	100
Beta erythroidine hydrochloride	5.0	2	0	35.0	9	56	100.0	3	0
	7.5	3	33	40.0	12	42	150.0	8	25
	10.0	3	100	50.0	9	89	175.0	8	75
							200.0	3	100

than for the alkaloids by these two routes. The surely fatal dose of quinine ethochloride dihydrate was 50 mgm. per kilo or about eight times that given intravenously; quinine methochloride dihydrate required 100 mgm. per kilo which is 10 times the dose by the intravenous route. Surely fatal doses for dihydro-beta-erythroidine and beta-erythroidine were 37.5 mgm. and 200 mgm. per kilo, or approximately three and four times the intravenous dosages.

*Gastric Absorption.* Gastric absorption was determined in dogs. Food was

withdrawn from all animals the night before the day of administration of the drugs. The dose was given either in capsule form or as a watery solution by stomach tube. Data on gastric administration are presented in table 2. It can be seen that all of the agents are effective by this route and the order of effectiveness is the same as that for toxicity. No evidence of severe gastric discomfort was observed with any of these agents. However, after absorption the erythrina alkaloids gave the usual gastrointestinal and cardiac symptoms already mentioned.

*The Anticonvulsant Action.* A total of 77 experiments were carried out on 34 dogs. If any animal was used more than once, a rest period of 24 to 72 hours was allowed before subsequent experiments. The routine of drug administration was as follows: the curare-like agent was injected rapidly in from 15 to 25 seconds and one minute allowed for development of the paralytic effect. Metrazol was then administered and the extent of the response, its duration and the recovery time of the animal noted. The intensity of the curariform action was graded as zero (0) if there was no modification of the convulsive seizure to four plus (+++) which represents almost complete absence of muscular spasms, but not necessarily fatal paralysis. Artificial respiration, when employed, was maintained by tracheal intubation. This was not induced until 4 minutes had elapsed from the time the curare-like agent was given. It was observed that if an animal did not attempt spontaneous diaphragmatic breathing between the 4th and 6th minute, survival without assistance was not assured. In certain experiments no attempts were made to assist the animal regardless of its condition. This was done to test maximum degree of paralysis and, incidentally, protection which could be obtained and still permit spontaneous recovery of the animal. Recovery time is represented by the time period elapsing between the beginning of the injection of the paralytic agent to that stage of recovery where the animal was able to stand without assistance.

The curare-like agents were used as 1 or 2% solutions in 0.9% saline. Metrazol was used as a 10% aqueous solution. All drugs were administered by vein. The optimal convulsant dose of metrazol as tested in 25 dogs was found to be 25 mgm./kg. The convulsive seizure appeared between the 6th and 7th second after administration and persisted for from 1 minute 45 seconds to 3 minutes 25 seconds with complete recovery in 2 to 5 minutes. The dosages of the curare-like agents varied since it was planned to ascertain the amount of drug required to give maximum protection against the standard metrazol dose without fatal paralysis. Details are given in table 3.

All of the curare-like agents modified the metrazol seizures. Quinine ethochloride gave the best protection without complicating respiratory paralysis. Eight and one-half mgm. limited the muscular spasms to feeble clonic twitchings of the extremities. Diaphragmatic breathing usually began in 5 minutes and recovery time averaged 13 minutes. Higher doses afforded no more protection but produced complete respiratory paralysis which necessitated artificial respiration to save the animal. Quinine methochloride was less effective and required amounts approaching the fatal paralytic dose. The protective dose for dihydro-

TABLE 2  
Gastric absorption

SUBSTANCE	DOSE	NUMBER OF ANIMALS	ONSET OF PARALYSIS	FATE OF ANIMALS
Quinine ethochloride dihydrate	mgm. per kgm.	10	minutes	No indications of paralysis
				No indications of paralysis
				Death 2 hours after onset of paralysis
				Death 2 hours after onset of paralysis
				No indications of paralysis
				Death 2 hours after onset of paralysis
				Death 26 minutes after onset of paralysis
				Death 13 minutes after onset of paralysis
Quinine methochloride dihydrate	200 to 300	5	35	No indications of paralysis
				Death 2½ hours after onset of paralysis
				Recovery 1½ hours after onset of paralysis
				No indications of paralysis
				Recovery 3 hours 35 minutes after onset of paralysis
				Death 36 minutes after onset of paralysis
Dihydro-heta- erythroidine hydrobromide	12.5	1	12	Recovery in 27 minutes after onset of paralysis
				Death in 20 minutes after onset of paralysis
				Death in 19 minutes after onset of paralysis
				Death in 30 minutes after onset of paralysis
				Death 20 minutes after onset of paralysis
				Death 20 minutes after onset of paralysis
Beta-erythroidine hydrochloride	25	1	9	Recovery 5 minutes after onset of paralysis
				Recovery 15 minutes after onset of paralysis
				Death 36 minutes after onset of paralysis
				Death 16 minutes after onset of paralysis
				Death 11 minutes after onset of paralysis
				Death 13 minutes after onset of paralysis

beta-erythroidine appeared to be between 1.5 and 2 mgm. With higher doses muscular twitchings appeared similar to those seen in nicotine poisoning even before the metrazol was introduced. Beta-erythroidine gave similar responses. In view of the unsatisfactory reactions obtained and the appearance of severe

TABLE 3  
Anticonvulsant action

DRUG	NUMBER OF EXPERIMENTS	DOSE	DEGREE OF PROTECTION	TIME FOR RECOVERY	REMARKS
		mgm. per kgm.		minutes	
Quinine etho-chloride dihydrate	1	5.0	+	2	
	2	7.5	+	2-3	
	8	8.5	++++	8-18	
	17	9.0	++++	6-15	4 animals required artificial respiration
	9	10.0	++++	5-22	5 animals required artificial respiration for survival
	3	12.5	++++	8-18	All animals required artificial respiration
Quinine metho-chloride dihydrate	3	7.5	+	4	
	5	10.0	+++	9-11	
	3	12.5	++++	12-15	One animal died
	3	15.0	++++		All animals died of cardiac arrest in 4 minutes
Dihydro-beta-erythroidine HBr	1	0.5	0	5	
	1	0.75	+	15	
	4	1.0	++	15-25	Artificial respiration in 2 animals
	2	1.5	+++	16	1 animal died despite artificial respiration
	1	2.0	+++	26	Artificial respiration required
	1	3.0	+++	30	Artificial respiration required
	1	5.0	+++		Animal died despite artificial respiration
Beta-erythroidine HCl	2	4.0	+	6-12	
	2	5.0	+++	18-25	Both animals required artificial respiration for survival

side effects with these alkaloids the number of experiments carried out was not large.

*The Cardiac Effects.* No untoward cardiac effects were noted after the administration of the quininc derivatives. The actions of the erythrinc alkaloids on the heart, however, were of some significance since Williams (4) has reported one death due to cardiac failure which he ascribes at least in part to beta-erythroidine. The side effects as noted here had all the indications of a marked para-

sympathetic stimulation. Since the alkaloids are quaternary ammonium bases (5) it is possible that a nicotine or muscarine-like action might be responsible for the irregularities. Accordingly, atropine was tried in a number of experiments and it proved effective in preventing the cardiac slowing and salivation, but not the gastrointestinal symptoms. Further evidence of the parasympathetic response was obtained by irrigating the exposed turtle heart with a solution of the alkaloids. It was found that vagus stimulation became ineffective after application of a 1% solution of these alkaloids. In a second series of experiments 7 normal dogs were treated with dihydro-beta-erythroidine, 1 mgm., before and after vagotomy. The average normal heart rate of the group was 135, after dihydro-beta-erythroidine, 64. Three hours after vagotomy, performed under ether anesthesia, the average rate was 201. The alkaloid, under these conditions, slowed the heart to an average of 152 beats per minute. Vagal stimulation was relatively ineffective. From these observations it is seen that apparently part of the cardiac slowing is produced by either a peripheral nicotinic ganglionic or muscarine action, with little or no central vagal stimulation.

*Discussion.* Certain general statements may be made with reference to the curare-like properties of these agents. All of them possess marked curariform actions. Within certain limits dihydro-beta-erythroidine is the most potent. Large doses do not intensify the paralysis. Muscular twitchings about the eyes and ears have been noted and these may spread to other parts of the body. Undesirable side actions on the heart and gastrointestinal tract have already been mentioned. Beta-erythroidine has about one-fifth the potency of the dihydro derivative, but otherwise the action of the two alkaloids is identical. The toxic manifestations of these alkaloids seem to preclude their general recommendation in psychiatric practice.

Quinine methochloride is one of the least active compounds in the group. The impression is, however, that once the paralytic effect is established there is a more prolonged action than with the other agents. Quinine ethochloride appears to have the greatest latitude of safety. The anticonvulsant dose is about one-half the minimal fatal dose. Those animals receiving large doses could always be saved with artificial respiration whereas a number of deaths occurred with the other agents under similar conditions. It is possible that the ethyl ester is the least stable and the rapid break-down in the body may be a factor in the rapidity with which the effects wear off.

Attempts were made to determine whether prostigmine would antagonize the curariform paralysis of any of these agents and thereby hasten recovery time. In 11 experiments prostigmine was given intravenously in doses of 0.05 mgm. 4 minutes after introducing the paralyzant. No significant difference was noted. Artificial respiration is still the best antidote.

#### CONCLUSIONS

dil

, quinine methochloride  
and beta-erythroidine

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	1	2.0	+++	26	Artificial respiration required
	1	3.0	+++	30	Artificial respiration required
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# AN IN-VIVO METHOD FOR THE EVALUATION OF GERMICIDAL SUBSTANCES USED FOR SKIN DISINFECTION<sup>1,2</sup>

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It is a common practice to evaluate the power of a germicide to kill some specified strain of bacteria by the *in vitro* determination of its phenol coefficient. Such a method has long been known to have certain limitations. Two most important factors in the evaluation of a germicide are its ability to penetrate the system present, and to act in the presence of tissue. It was with these things in mind that work was undertaken to determine the value of certain germicidal substances under actual *in vivo* conditions.

In work as yet unpublished the writer found that the germicidal action of certain widely used preparations, when used in the presence of tissue, showed values that were lower than their respective values when determined on the basis of the phenol coefficient test. A procedure was desired that would measure the ability of the germicide to act in the system actually present. Kempf and Nunester (4) have suggested an *in vivo* method in which a piece of contaminated mouse tail is treated with a germicide and placed in the peritoneal cavity of a white mouse. We have attempted to standardize and modify this procedure to fit our needs.

**EXPERIMENTAL.** In brief, this experiment consisted of applying to the slightly abraded skin of white mice, organisms capable of causing a fatal peritonitis. A germicide was then applied to the area and after a set time the infected skin was inserted into the peritoneal cavity. If the germicide had killed the bacteria the mouse showed no ill effects, but if not, a fatal peritonitis followed. The method is described later in greater detail.

The action of the germicide was tested in the presence of both functioning and non-functioning tissue—the latter term referring to tissue in which normal function has been interrupted by the death of the animal—in the belief that there might be a difference between the two physiological states.

**Test germicides.** The following four germicides were picked for comparison because they are representative of four classes of compounds widely used in the field of disinfection and because the first three are at present widely used in clinical medicine.

Tincture of Iodine (U.S.P.)

Sodium Ethyl Mercury Thiosalicylate, 1:1000 tincture

Hexylresorcinol, 1:1000 aqueous solution

Cetylpyridinium Chloride,<sup>3</sup> 1:200 tincture

**Bacterial culture.** The infecting strain used in these experiments was the *Streptococcus hemolyticus*, chosen because it is found on the skin and is a constant producer of peritonitis,

<sup>1</sup> Presented at the meetings of the Society of American Bacteriologists, Baltimore Maryland, December 30, 1941.

<sup>2</sup> The investigation herein reported was made possible through support granted by The Wm. S. Merrell Co., Cincinnati, Ohio.

<sup>3</sup> Cetylpyridinium Chloride supplied by the Wm. S. Merrell Co., Cincinnati, Ohio.

septicemia, and pyogenic infections. An original culture was enhanced in virulence by direct serial passage through the peritoneal cavities of white mice, until, of 0.5 cc. of physiological saline solution injected into the peritoneal cavity of a dead infected mouse, only 0.1 cc. of the resultant suspension was necessary to cause death by peritonitis in 6 to 8 hours. This passage was continued throughout the time of the experiments and cultures used were peritoneal exudate cultured onto Bacto brain-heart infusion agar slants containing 10% defibrinated sheep blood and incubated for 24 hours at 37°C. Five cc. of physiological saline solution were then added to make the suspension for the test.

*Testing of germicides on functioning skin.* An 18 to 22 gram white mouse was etherized and placed on its back on a board by means of adhesive tape. The abdominal area was lathered, shaved, and the residual soap washed off with 70% alcohol. On this surface, an area one-half inch square was marked off by four dots of tincture of iodine. Six slight cross-scratchings were made with a needle in the designated area. A 4 mm. wire loop filled with a suspension of the test organism was gently massaged into this area for one minute, after which the entire abdominal area was painted with the germicide to be tested. Three minutes were allowed for its action, after which time the designated area was aseptically excised, the peritoneal cavity opened, and the piece of skin placed inside. After closure the exterior surface of the wound was lightly painted with tincture of iodine. The animals were observed for a period of seven days. In case of death they were autopsied and microscopic and cultural examinations were made of the peritoneal exudates. The above procedure was not followed until control experiments had demonstrated that the animals would not die in and of the operative procedure itself. On each day all germicides tested were used on an equal number of animals to eliminate any discrepancy in the final results due to a possible day to day variation in either virulence or concentration of the test organism suspension. Against each day's quota of animals used experimentally an equal number of animals were subjected to all the other procedures with application of the germicide omitted.

*Testing of germicides on non-functioning skin.* A white mouse was killed and placed on its back on a board. After a waiting period of one-half hour, the abdomen was shaved and all residual soap removed with 70% alcohol. An area one inch square was indicated by means of four small dots of tincture of iodine. Twelve cross-scratches were made each way in the designated area. The test organism suspension was then massaged onto this area for one minute. The entire abdominal area was then painted with the germicide to be tested. After a period of three minutes the skin of the designated area was aseptically excised and divided into four equal pieces. Each section of skin was then inserted into the peritoneal cavity of another mouse under aseptic conditions, the incision closed, and the exterior surface painted with tincture of iodine. In this procedure, as in the preceding technique, on each day all germicides tested were used on an equal number of animals. The observation period and method of autopsy were identical with those employed in the former procedure, as was also the perfection of the technique. Against each day's quota of animals used experimentally, an equal number were subjected to all the other procedures with application of the germicide omitted.

**RESULTS.** In these experiments attempt was made to show the killing power of various germicides under *in vivo* conditions and to obtain evidence that a diffusion by the germicide through the disrupted surface tissues occurs. The scratching of the skin was for the purpose of presenting a substrate upon which this later action could occur. The time of three minutes which was given to the germicide to act upon the organisms and effect sterilization, is at least as long as is given in usual clinical procedures.

Table 1 shows the relative values of the four germicides tested. It will be noticed that all of the control animals died. At autopsy the peritoneal exudates

of all dead animals contained large numbers of gram positive hemolytic streptococci. Tincture of iodine offered the best protection against infection by saving 24 out of 25 animals. Cetylpyridinium chloride was next in value, protecting 14 out of 25 animals. Sodium ethyl-mercury thiosalicylate followed by saving 12 of the 25 animals, while hexylresorcinol saved but 9 out of 25 animals. The germicidal action of the alcohol-acetone tincture used in sodium ethyl-mercury thiosalicylate and cetylpyridinium chloride, was also tested against a series of animals and gave protection below either of these two substances. Many clinicians object to the use of tincture of iodine in surgery be-

TABLE 1

*The germicidal action of various substances in the presence of functioning tissue*

GERMICIDE	NUMBER TESTED	NO. OF ANIMALS LIVING IN X DAYS						
		1	2	3	4	5	6	7
Iodine (tinct.) . . . . .	25	24	24	24	24	24	24	24
Sodium ethyl-mercury thiosalicylate (1:1000 tinct.) . . . . .	25	13	13	12	12	12	12	12
Hexylresorcinol (1:1000 soln.) . . . . .	25	20	18	14	10	9	9	9
Cetylpyridinium chloride (1:200 tinct.) . . . . .	25	18	16	15	14	14	14	14
Control . . . . .	25	1	0	0	0	0	0	0

Autopsy: Positive. Hemolytic streptococci found in peritoneal exudate of all dead animals.

TABLE 2

*The germicidal action of various substances in the presence of non-functioning tissue*

GERMICIDE	NUMBER TESTED	NO. OF ANIMALS LIVING IN X DAYS						
		1	2	3	4	5	6	7
Iodine (tinct.) . . . . .	24	22	22	21	21	21	21	21
Sodium ethyl-mercury thiosalicylate (1:1000 tinct.) . . . . .	24	17	13	11	11	9	9	9
Hexylresorcinol (1:1000 soln.) . . . . .	24	11	10	10	9	8	8	8
Cetylpyridinium chloride (1:200 tinct.) . . . . .	24	14	12	12	11	11	11	11
Control . . . . .	24	0	0	0	0	0	0	0

Autopsy: Positive. Hemolytic streptococci found in peritoneal exudate of all dead animals.

cause of fumes, skin sensitivity, and color (5). Also, there are certain uses for germicides in which tincture of iodine is unsatisfactory for obvious reasons. It is in such cases that some less irritating preparation is desired. Therefore representatives of three widely used types of compounds, known to be less irritating, were employed, and tincture of iodine used for comparison.

Comparison of table 2 with table 1 presents a quite different picture. In the former, the germicidal action was on bacteria in the presence of tissue that had apparently ceased to function. The relative efficiencies of the four germicides

were the same as in the previous test, but the actual efficiencies were definitely lower although hexylresorcinol was about as effective in both cases. However, its efficiency was hardly high enough to make a comparison of definite value in either case. It appears therefore that there was some difference in the action in experiments 1 and 2 and it seems possible that whether or not the skin was in a state of normal function was the determining factor. Colebrook (3) found that hemolytic streptococci are rapidly killed on the skin of the normal hand. Three minutes after swabbing a finger lightly with a broth culture—by which time the skin was apparently dry—30,000,000 cocci could be recovered by thorough swabbing with sterile broth. One hour later, 1,700,000 viable cocci could be recovered, and two hours later, 7,000 cocci. Control experiments eliminated desiccation as the cause of this reduction. Similar observations were made with *Proteus vulgaris*, *Friedlander's pneumobacillus*, and *Escherichia coli*. Arnold, *et al.* (1), have shown that the dead skin, tested on cadavers within 15 minutes of death, had lost most of its bactericidal action.

In toxicological studies made by Warren *et al.* (2) cetylpyridinium chloride was shown to exhibit its highest toxicity when administered by the intraperitoneal route. Since our procedure risked the intraperitoneal introduction of a considerable amount of cetylpyridinium chloride on the inserted piece of skin, it was desired to determine if a sufficient amount might be introduced in the experimental procedure to cause toxic symptoms and possible death. Therefore the procedure was carried out using the cetylpyridinium chloride generously and omitting the test organisms. In the nine animals so tested, none exhibited the typical curare effect or other signs of discomfort, and therefore, it was concluded that a toxic quantity of cetylpyridinium chloride was not being introduced.

#### SUMMARY AND CONCLUSIONS

1. Several germicidal substances have been tested by a method giving actual *in vivo* conditions. This method is believed to be more reliable in determining the comparable germicidal efficiencies than is an *in vitro* method.

2. Cetylpyridinium chloride has been shown to be non-toxic when used in this test.

3. Representatives of four widely used classes of germicidal compounds have been tested. Tincture of iodine proved to be very high in germicidal efficiency when evaluated by this method. In tests conducted on a group of non-irritating germicidal compounds, a quaternary ammonium compound, cetylpyridinium chloride, showed the highest relative efficiency. Sodium ethyl-mercury thiosalicylate and hexylresorcinol followed in effectiveness in the order given.

4. Evidence has been advanced to support the theory that the skin has a definite bactericidal power which it may exert along with chemicals so that the two together may overcome a bacterial invasion or infection that neither the skin alone nor the germicide alone were able to do in the experimental work.

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# THE EFFECT OF MUSCLE EXTRACTIVES ON THE PERFUSED HEART

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Because of the increasing interest in the production of experimental shock by such methods as traumatization of muscle and implantation of tissue in the peritoneal cavity, we decided to study the pharmacologic effects of some of the various compounds which might be liberated in such procedures. Flossner (3) and du Vigneaud and Behrens (2) noticed some pertinent circulatory effects following intravenous injection of aqueous muscle extracts into intact animals, including lowering of blood pressure and bradycardia.

Due to the difficulty in analyzing for the multitudinous substances liberated from traumatized or autolyzing muscle, we chose to use a commercial aqueous extract of beef muscle, since considerable information concerning its chemical constituents was available by courtesy of the company's chemists. It may be assumed that at least some of the constituents of this extract are found also in fresh extracts of muscle. Moreover, such a standard preparation is readily available. The extract used (Valentine's Meat Extract) is made by extracting beef muscle with hot water and concentrating it *in vacuo*. It contains only a trace of heat-coagulable protein.

**METHODS** The isolated frog heart preparation perfused through the sinus venosus under constant pressure was used, as described by Spealman (7). Because of the high concentration of potassium in this extract (0.728 M), it was necessary to use K-free Ringer's in making the various dilutions used, except when such low concentrations of extract were employed that insufficient K would be present. In such cases, 0.1% extract and below, sufficient KCl was added to bring the K content up to normal Ringer's solution, as calculated from the K content of the extract. The formula of our normal frog Ringer's solution is: NaCl 0.10 M, CaCl<sub>2</sub> 0.002 M, KCl 0.002 M, NaHCO<sub>3</sub>, 0.002 M. Also, since the pH of the extract was 5.6, the pH of the final dilution was always brought to 7.6 by addition of NaOH, as measured with the glass electrode.

The frog heart was perfused with normal Ringer's solution until the rate and amplitude of the beat was stabilized. This usually required 30 to 40 minutes. The experimental Ringer's containing varying concentrations of meat extract was then substituted and the effects observed for one hour. Then normal Ringer's was again perfused for 30 minutes, to redetermine normal rate and amplitude.

To obviate the tendency of friction of the heart lever on smoked paper to simulate a reduction in amplitude of contraction, the excursion of the lever was measured by observing the movement of the heart lever over a millimeter scale, and the rate was determined with a stop watch, as recommended by Spealman (9). These measurements were taken every 10 minutes during the course of the entire experiment.

**RESULTS.** To our surprise, this muscle extract in proper dilution consistently produced a marked increase in amplitude of the beat (fig. 1) instead of the

weakening or slowing we had expected. This positive inotropic effect was maintained as long as the extract was perfused, the experiments sometimes lasting for 90 minutes. Fig. 2 illustrates in graph form typical results of an experiment in which an 0.4% extract dilution was used. We therefore dropped the original purpose of this investigation in favor of determining the nature of this stimulating substance.

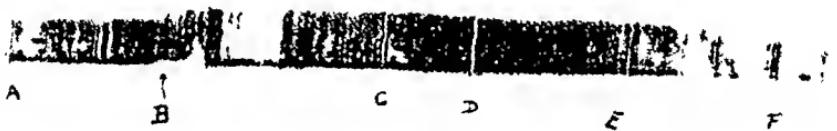


FIG. 1. EFFECTS OF MEAT EXTRACT IN K-FREE RINGER (0.4%) ON THE PERFUSED FROG HEART

- A-B. Last 3 minutes of a 30 minute perfusion with Ringer.
- B. Perfusion fluid changed to meat extract in K-free Ringer (0.4%).
- B-C. First 5 minutes of perfusion with extract dilution.
- C. No recording for 10 minutes.
- C-D. Two minutes recording.
- D. No recording for 10 minutes.
- E. End of 30 minutes perfusion with extract dilution. Ringer substituted for extract dilution.
- E-F. First 4 minutes perfusion with Ringer.
- F. Ten minutes no recording.

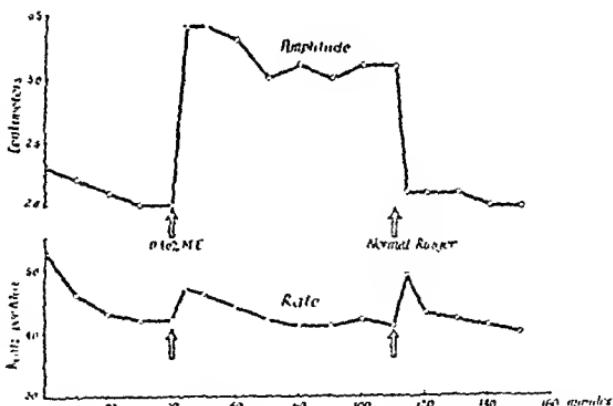


FIG. 2. EFFECT OF PERFUSING A 0.40% MEAT EXTRACT (S.E.) SOLUTION ON THE RATE AND AMPLITUDE OF CONTRACTION OF AN ISOLATED FROG HEART  
The arrows indicate the beginning and the end of meat extract perfusion

Table 1 shows the results obtained by perfusing the frog heart with various dilutions of meat extract, and indicates that dilutions in Ringer's ranging from 0.56 to 0.05% exert a positive inotropic effect. Stoppage of the heart in diastole in the stronger concentrations is probably due to the unavoidable excess K of the extract. The use of K-free Ringer's as diluent for concentrations below 0.1% extract also resulted in stopping of the heart, probably due to lack of

sufficient K. Speelman (9), who has used this technic and has determined the approximate limiting physiologic concentrations of K for the frog's heart to be  $4 \times 10^{-3}$  M to  $1.0 \times 10^{-2}$  M, has made similar observations. Within these limits, K does not appreciably affect the amplitude of contraction (5).

Since there was often a slight change in rate of the heart, usually a slowing, which might of itself alter the amplitude of contraction, several of the effective concentrations of meat extract were tested on perfused hearts driven at the constant rate of 40 per minute by electrical stimulation. This procedure did not affect the positive inotropic effect of the extract.

In order to determine whether this effect could be obtained in the mammalian heart, similar dilutions in K-free mammalian Ringer's were tested by the method of Speelman (9) on several isolated guinea pig atria. The results were similar

TABLE 1

*The effect of various concentrations of meat extract on the isolated frog heart*

Potassium was added to the solutions marked\* to bring the concentration of this ion into the physiologic range. "S" indicates stopping of the heart, usually within 10 minutes. "0" indicates no change.

MEAT EXTRACT CONCENTRATION cc. per 100 cc.	RATE CHANGE		MEAT EXTRACT CONCENTRATION cc. per 100 cc.	RATE CHANGE	
	per cent	per cent		per cent	per cent
1.00	S	S	0.29	-27	+43
0.67	S	S	0.22	-7	+20
0.67	S	S	0.20	-10	+11
0.56	S	S	0.10	-5	+10
0.56	+2	+50	0.10	0	+32
0.50	-9	+67	0.05	+12	+7
0.40	-7	+63	0.05	0	+22
0.40	0	+55	0.02	0	0
0.40	+7	+56	0.02	0	0
0.40	-12	+44			

to those obtained in the frog heart, a positive inotropic effect always being obtained.

Experiments were then undertaken to determine the nature of the substance producing this positive inotropic effect. Table 2 summarizes the results. Dialysis was performed by placing 4 cc. of the meat extract in a segment of cellophane tubing (Visking sausage casing) and suspending it in 400 cc. distilled water for 12 hours at room temperature, with gentle stirring. The fluids inside and outside of the tube were diluted to 1:250 for testing, assuming that each contained 4 cc. of extract. K-free Ringer was used to dilute the outside solution since K is freely dialyzable, while normal Ringer was used for the solution inside. Table 2 shows that this active substance was easily demonstrated in the water outside the tube and to a much less degree inside, indicating that it is dialyzable.

Electrolysis was performed by placing 5 cc. of meat extract in a U-tube and

employing platinum electrodes immersed in saline, 7 cm. apart, through which a 5 to 7 volt current was passed at 1 to 2 milliamperes over a period of about 20 hours. The liquid about each electrode was then drawn off and tested on the heart in the usual way. K-free Ringer was used to dilute the cathode solution because of accumulation of K, and normal Ringer was used for the anode solution. The pH was adjusted in each case. As table 2 shows, the active substance went to the anode when the extract was at pH 5.6, indicating that it was negatively charged at this pH. To determine if this charge could be reversed by lowering the pH, electrolysis was carried out at pH 2. The active substance did not appear at either pole at this pH. However, a substance hav-

TABLE 2  
*Properties of the active fraction of meat extract*

"0" indicates no change. Each set of rate and amplitude values was obtained on a separate frog heart. For further explanation see text.

SOLUTION STUDIED	RATE CHANGE	AMPLITUDE CHANGE	SOLUTION STUDIED	RATE CHANGE	AMPLITUDE CHANGE
Dialyzed fraction	per cent	per cent	Cath. sol. (pH 2.0)	per cent	per cent
	+4	+56		+12	0
	-40	+23		+7	-21
Non-dialyzable fraction	0	+41		0	-20
	+25	0	Alcohol sol. fraction	+6	+69
	-4	+5		-47	+22
Anode sol. (pH 5.6)	0	+15		0	+57
	+14	+22	Alc. resid. fraction	+10	0
	+18	+30		+21	-6
Cath. sol. (pH 5.6)	-26	0		+11	+41
	+21	-11		+7	+19
	+13	+33		0	0
Anode sol. (pH 2.0)	0	0	Ether sol. fraction	0	0
	0	0		0	0
	0	0		0	0

ing negative inotropic properties appeared at the cathode, which may have masked the presence of the other substance.

To determine the alcohol and ether solubility of this substance, alcohol was added to the extract to make a 70% alcohol solution. The insoluble material was centrifuged off and dissolved in normal Ringer's to form the *alcohol-residue fraction*. The alcohol was boiled from the supernatant fluid at 95°C., and the remainder was then made up to volume with K-free Ringer to form the *alcohol-soluble fraction*. Table 2 indicates the presence of the active substance in both alcohol-soluble and alcohol-residue fractions, indicating some solubility in 70% alcohol and stability at 95°C.

Ether solubility was tested by shaking the extract with ether and testing the two layers, after removing the ether by evaporation and making up to volume with normal or K-free Ringer as indicated. Table 2 shows that this substance is insoluble in ether.

With these data concerning the behavior of the active ingredient, a number of pure compounds reported to be in this meat extract, and having somewhat similar chemical properties, were dissolved in Ringer and tested separately on the isolated frog heart. These substances were: histamine, adenine, xanthine, guanine, uracil, *d*-arginine, methyl guanidine, creatine, and creatinine. They were tested in concentrations between 0.0001% and 0.10%. Other substances such as hypoxanthine and carnosine were not tested, due to lack of information concerning their presence in this extract. The only ones of this series having a similar effect on amplitude in the concentrations tested were guanidine and creatine.

TABLE 3  
*The effect of creatine on the isolated frog heart*

HEART NUMBER	CREATINE CONC. gm. per 100 cc.	RATE CHANGE per cent	AMPLITUDE CHANGE per cent	REMARKS
1	0.10	0	+20	Response delayed 30 min.
2	0.10	+7	0	Depression, followed by rise
3	0.0008	0	+25	Response delayed 30 min.
3	0.0025	+18	+50	Immediate response, but same heart as above
4	0.0022	0	+26	Delayed for 20 min.
5	0.0020	-6	+15	Delayed for 30 min.
6	0.0020	0	0	No response
7	0.0100		+33	Electrically paced at rate of 40 per min.
8	0.0100		0	Electrically paced at rate of 40 per min.
9	0.0020		+21	Electrically paced at rate of 40 per min.
10	0.0020		0	Electrically paced at rate of 40 per min.

Since guanidine is present in but very small quantities in this extract, it was not considered further. However, the extract does contain 1.02 gm. creatinine and 0.79 gm. creatine per 100 gm. extract. Backman (1) found a positive inotropic effect of creatine on the isolated rabbit heart, and Shapiro (6) has demonstrated a similar action of both creatine and creatinine on perfused toad, rabbit and cat hearts, although both used higher concentrations (0.1-0.2%) than could be present in our effective extract dilutions. Since we found creatinine ineffective in concentrations in which it is present in the dilutions of extract used, we believe that creatinine is not the important factor in meat extract.

However, table 3 shows that creatine, in most of the hearts tested, has a definite positive effect on amplitude, although somewhat less than the extract, between concentrations of 0.0008% and 0.1%. Sometimes, however, this effect of creatine is delayed for as long as 30 minutes, a phenomenon we have

never observed when using meat extract. It is of interest that the lower effective concentrations approach the concentration of creatine in human blood (3 to 7 mg. per 100 cc.).

From the effective concentrations of extract in table 1, it appears that the amounts of creatine present were adequate to explain in part their inotropic effect. For example, the 0.1% extract dilution, which is distinctly effective on the heart, would contain about 0.0008% creatine, which can be effective alone. Moreover, the chemical properties of this active substance resemble those of creatine. As further evidence that creatine may be concerned, autoclaving the extract at pH 2 for 30 minutes under 15 lb. pressure, destroys its activity completely. Such treatment should change all the creatine to creatinine (4). The differences between the effects of the extract and of creatine may be due to the additive effect of the other compounds in the extract, in concentrations which are below threshold when tested singly.

#### CONCLUSIONS

A commercial meat extract, diluted with normal or K-free Ringer and with pH adjusted, has a marked positive inotropic effect upon the isolated perfused frog heart and on the guinea pig atrium. The active substance is soluble in water and 70% alcohol, is ether insoluble, moves to the anode at pH 5.6, is stable at 95°C., but is destroyed by autoclaving at pH 2. Of the several known substances in meat extract tested, creatine resembles this most closely, since in similar concentrations it has a positive although sometimes delayed inotropic effect on most of the hearts tested, has similar chemical behavior, and is present in the meat extract in concentration adequate to explain in large part this pharmacologic effect. The inotropic effect of creatine is often demonstrable in concentrations approaching those in human blood.

We are indebted to Dr. W. A. Peabody of the Valentine Meat Juice Company for the K analyses and information concerning the ingredients of the meat extract used, as well as for the supply of extract.

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# PHARMACOLOGICAL AND TOXICOLOGICAL STUDIES ON *L*-*N*-ETHYLEPHEDRINE HYDROCHLORIDE<sup>1,2</sup>

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Curtis (1), in 1929, reported some work of a preliminary nature on the action of a number of tertiary amines related to ephedrine. The object of his investigation was to determine whether these new compounds were more suitable for therapeutic purposes than *L*-ephedrine. Later Pak and Read (2, 3) carried out extensive work with *L*-*N*-methyllephedrine and compared its action with that of *L*-ephedrine. From their results they concluded that methylation of *L*-ephedrine diminishes many of the actions of this compound but that the effect on the bronchioles is about the same in degree although somewhat slower.

In the present paper a study of the effect of ethylating *L*-ephedrine is reported.

**CHEMICAL AND PHYSICAL PROPERTIES** The *L*-*N*-ethylephedrine used in the following experiments was synthesized in this laboratory. Although the levo and racemic forms were prepared, only the results obtained with the levo form are reported.

*L*-*N*-Ethylephedrine hydrochloride, a tertiary amine known chemically as levo-*L*-phenyl-*2*-methyl-*2*-ethylethylaminopropan-*1*-ol hydrochloride, is a white crystalline, odorless compound. Its solubility is 70 grams per 100 cc. in water and 14.1 grams per 100 cc. in ethyl alcohol. The molecular weight is 229.5 and the melting point 183-184°C. This compound is very stable and solutions of it are not decomposed following exposure to light and air.

**RESULTS OF EXPERIMENTAL STUDIES.** In these studies the hydrochlorides of the various compounds were used. Unless otherwise stated all doses were calculated as mgm. per kgm.

**Toxicity.** Two types of tests were conducted to determine the toxicity of *L*-*N*-ethylephedrine—one to determine the acute toxic effects, and the other to observe the effect of prolonged administration of large doses. In both instances observations were made following oral and intravenous administration of the compound.

Rabbits were used as the test animal in the *acute toxicity studies*. All intravenous injections were made into the marginal ear vein at the rate of 50 mgm. per minute. In the oral toxicity studies a 10% solution was administered by stomach tube. Lethal or slightly less than lethal doses of *L*-*N*-ethylephedrine produced definite symptoms; an increase in the respiratory rate, an increase in the pulse rate and blood pressure, and some mydriasis and clonic convulsions. The animals either died during the convulsion or passed into a

<sup>1</sup> *L*-*N*-Ethylephedrine Hydrochloride ("Nethamine" brand) produced by The Wm. S. Merrell Company.

<sup>2</sup> Presented before the American Society for Pharmacology and Experimental Therapeutics, Inc., Chicago Meeting, April, 1941, (THIS JOURNAL, 72: 3, 44, 1941).

period of depression from which they either rapidly recovered or died. Death was due to respiratory and cardiac failure. Animals which received sublethal doses recovered without any complications. Similar symptoms were obtained in some simultaneous toxicity studies with *l*-ephedrine.

The results presented in table 1 show that *l*-*N*-ethylephedrine administered intravenously killed one out of 12 rabbits at a dosage level of 50 mgm. and approximately 50% at a dosage level of 65 mgm. In similar studies with *l*-ephedrine it was found that one out of 12 rabbits died at a dosage level of 55 mgm. The rate of injection in this series of tests was the same as that with *l*-*N*-ethyl-ephedrine. Orally *l*-*N*-ethylephedrine was tolerated in large amounts as shown in table 2. The acute toxic effects as well as the M.L.D. of *l*-*N*-ethyl-ephedrine and *l*-ephedrine are, therefore, very much the same.

TABLE 1  
*Acute intravenous toxicity—rabbits*

	DOSE: MG.M./KGM.					
	45	50	55	60	65	70
Number of animals dead.....	0	1	2	3	5	7
Number of animals injected.....	12	12	12	12	12	12

TABLE 2  
*Acute oral toxicity—rabbits*

	DOSE: MG.M./KGM.			
	540	550	560	570
Number of animals dead .. ..	0	2	3	4
Number of animals injected .. ..	12	12	12	12

The effect of *prolonged administration* of large amounts of *l*-*N*-ethylephedrine was also studied in rabbits. The animals were divided into the following groups: controls; a group receiving the compound in a daily dose of 25 mgm. administered intravenously; another, receiving this same amount orally; and a fourth, receiving a daily oral dose of 50 mgm. All the dosages in these tests were calculated on the basis of the weight of the animals at the beginning of the test period. Regardless of the mode of administration or of the dose, the animals received the compound daily for a period of four weeks. At the end of this time one-half of the animals in each group were sacrificed and autopsied. Tissues were kept for histological study. Two weeks after cessation of drug administration the remainder of the animals were killed and observations made as before. Weight records which were kept throughout the test period showed that practically all the animals gained weight.

Gross and microscopic examination of the tissue revealed no pathological changes which could be attributed to the administration of the compound. The rabbits in the group receiving 25 mgm. daily of *l*-*N*-ethylephedrine ad-

ministered intravenously actually received one-half a lethal dose daily or a total of 14 lethal doses throughout the test period. On the basis of these tests it may be concluded that in rabbits the daily administration of *L-N*-ethylephedrine in the above doses for a period of 4 weeks did not produce any tissue injury.

*Effect on the Circulatory System.* *L-N*-Ethylephedrine produced a characteristic effect on the circulatory system. This effect was qualitatively similar to that produced by *L*-ephedrine but differed quantitatively. *L-N*-Ethylephedrine, 1 mgm., administered intravenously to harbitalized dogs produced an 8 to 10 mm. mercury rise in blood pressure. This rise was equal to about one-half of that produced by a similar amount of *L-N*-methylephedrine and about one-tenth to one-fifteenth of that produced by a similar amount of *L*-ephedrine.

Since subsequent injections of any of these three compounds produce a decreasing effect it was difficult to obtain accurate comparative results of their pressor actions. A series of tests were therefore made in which the pressor action of each compound was compared with that of epinephrine hydrochloride. The method was essentially the same as that described by Chen (4). Pithed dogs were used and the blood pressure was recorded from the right common carotid. An average of the results indicates that 1 mgm. of *L*-ephedrine produced a rise in blood pressure 10 times greater than a similar amount of *L-N*-ethylephedrine and that *L-N*-methylephedrine produced a rise one-half as great as that produced by a similar amount of *L*-ephedrine, and five times as great as a similar amount of *L-N*-ethylephedrine. The results obtained with *L-N*-methylephedrine agree with those reported by Pak and Read (2). Characteristic results are presented in figure 1. If the amount of *L-N*-ethylephedrine was increased to 5 mgm. then the rise in blood pressure was equal to about one-half of that elicited by a similar amount of *L*-ephedrine.

The effect of *L-N*-ethylephedrine on the peripheral circulation was determined in frogs and dogs. The results of perfusion experiments on frogs indicated that it produced very little vasoconstriction. A few experiments were run on dogs using a nasal plethysmograph. Again there was very little indication of vasoconstriction of the nasal mucosa.

The effect of *L-N*-ethylephedrine on the pulse rate was determined in dogs. In the normal dog an intravenous dose of 1 mgm. of the compound produced no significant changes in the pulse rate. When the amount was increased to 5 mgm., a dose which produces a more marked rise in the blood pressure, there was a simultaneous decrease in the pulse rate which was of central origin since it was prevented with atropine sulfate.

In further tests the effect on the amplitude of cardiac contractions was determined. For these experiments dogs were anesthetized with morphine sulfate followed by chloroform. Heart tracings were obtained with a myocardiograph. The results indicated that the amplitude was slightly increased. In further tests an attempt was made to determine the site of action of *L-N*-ethylephedrine on the heart of dogs. Typical results are presented in figure 2. It is apparent that this compound stimulated the heart through the stellate ganglia and also at the peripheral nerve endings.

*Effect on Respiration.* The effect of *l*-*N*-ethylephedrine on respiration was determined on normal dogs. Doses of 1 mgm. administered intravenously to these animals produced an increase in the depth of respiration but had little or no effect on the rate. If the amount was increased to 5 mgm. there still was no consistent or significant change in the rate. The depth of respiration again was increased at this dosage level.

*Effect on the Bronchioles.* Experiments were conducted to determine the effect of *l*-*N*-ethylephedrine on the bronchioles. The method of Sollmann and Von Oettingen (5) was employed in some preliminary tests using rabbit, cat and guinea pig lungs. The results indicated that *l*-*N*-ethylephedrine produced as great a bronchial dilatation as *l*-ephedrine.

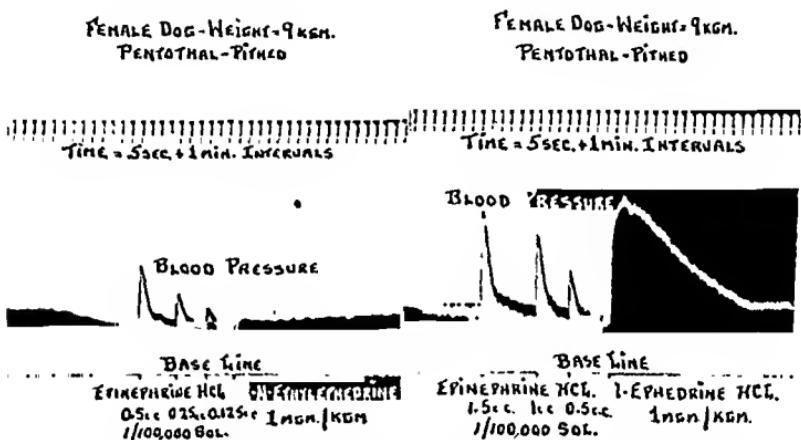


FIG. 1. BLOOD PRESSURE IN PITHED DOGS

The pressor effect of *l*-*N*-ethylephedrine or *l*-ephedrine compared with that elicited by epinephrine hydrochloride. *l*-*N*-Ethylephedrine (left tracing) 1 mgm./kgm. equivalent to 0.00014 mgm./kgm. of epinephrine hydrochloride and *l*-ephedrine (right tracing) 1 mgm./kgm. equivalent to 0.00166 mgm./kgm. epinephrine hydrochloride.

In further studies on decerebrate dogs the bronchial action was determined *in situ*. The method for recording bronchial contractions was the aspiration method described by Jackson (6). After a tracing of the normal respiratory excursions had been obtained bronchial constriction was produced in one of several ways: (a) arecoline hydrobromide, total dose of 0.3 mgm., (b) ergotoxine, total dose of 15-30 mgm. followed by 0.5 mgm. arecoline hydrobromide, (c) ergotoxine followed by 0.5 mgm. pilocarpine, (d) cocaine hydrochloride, total dose 100 mgm., followed by arecoline hydrobromide, and (e) histamine, 0.03 mgm. The compounds were all administered intravenously with the exception of cocaine which was given subcutaneously. All of these experiments indicated that *l*-*N*-ethylephedrine had the ability to produce bronchial dilatation to the same degree as *l*-ephedrine. However, in some tests it appeared that the action of *l*-ephedrine was of a somewhat longer duration. Typical results are presented in figure 3.

*Effect on the Intestine.* The effect of *l*-*N*-ethylephedrine on isolated strips of rabbit jejunum was determined and compared with that elicited by *l*-ephedrine. The strips were suspended in oxygenated Locke's solution at a constant tem-



FIG. 2. SITE OF ACTION—HEART

ne and chlorobutanol. A. applied *l*-*N*-ethylephedrine to amplitude of heart. B. applied *l*-*N*-ethylephedrine to *l*-*N*-use in amplitude. C. *l*-*N*-ethylephedrine removed. D. *l*-*N*-ethylephedrine removed at E. *l*-*N*-ethylephedrine applied to both ganglia at F and G—no effect on amplitude. H. *l*-*N*-ethylephedrine removed. I. *l*-*N*-ethylephedrine, 1 mgm./kgm., administered intravenously—increase in amplitudo of heart.

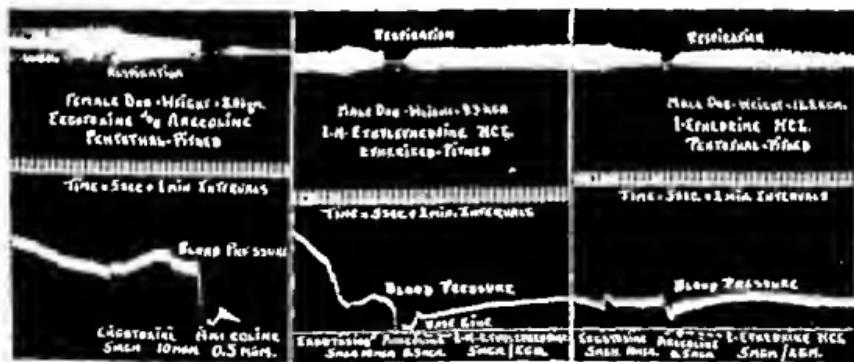


FIG. 3. BRONCHIAL ACTION IN PITHED DOGS

acholes and blood pressure. Left tracing: Re-  
of ergotoxine and 0.5 mgm. of arecoline hydro-  
ring a total dose of 10 mgm. of ergotoxine, 0.5  
m./kgm. of *l*-*N*-ethylephedrine. Right tracing:  
m. of ergotoxine, 0.5 mgm. of arecoline hydro-

— — — — — *atropine* or *epinephrine*.

perature of 38-39°C. Solutions of either compound in a concentration of 1:5,000 usually produced a relaxation of normal as well as of nicotinized jejunum. At times there was a definite stimulation of the normal intestine. Typical results are presented in figure 4. If sufficient atropine was added to

paralyze the parasympathetic nerve fibers and a spasm of the muscle was then induced by  $\text{BaCl}_2$  in either 1:2,500 or 1:5,000 solution, *l*-*N*-ethylephedrine produced a marked decrease in the tonus whereas *l*-ephedrine had only a very slight effect. In further tests muscle strips were nicotinized and a spasm was then induced by a 1:70,000 solution of pilocarpine. *l*-*N*-Ethylephedrine in a concentration of 1:5,000 again produced a marked drop in tonus. The results of all of these tests indicated that *l*-*N*-ethylephedrine usually produces a relaxation of intestinal muscle and that this action is somewhat greater than that produced by similar amounts of *l*-ephedrine.

A few preliminary experiments were carried out in which the effect was studied on the stomach *in situ*. The results suggest that *l*-*N*-ethylephedrine produces an inhibition of gastric peristalsis but further tests will be necessary before any definite statement can be made.

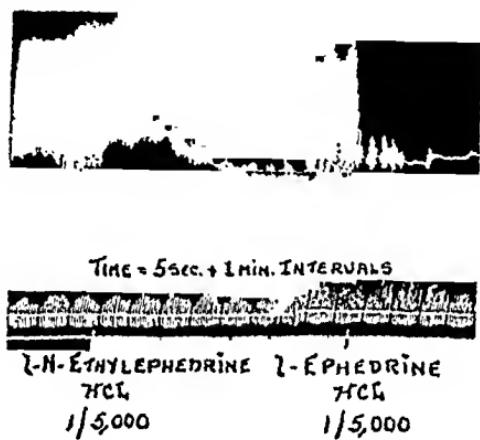


FIG. 4. COMPARATIVE EFFECT OF *l*-*N*-ETHYLEPHEDRINE AND *l*-EPHEDRINE ON THE ISOLATED INTESTINE OF RABBITS

*Effect on the Uterus.* Isolated strips obtained from virgin guinea pigs and rabbits were used. They were suspended in oxygenated Locke's solution and kept at a constant temperature of 38-39°C. The effect on the uterus was compared with that elicited by *l*-ephedrine and *dl*- $\beta$ -phenylisopropylamine. *l*-*N*-Ethylephedrine in a concentration of 1:5,000 produced a marked increase in uterine tonus with a diminution of the individual contractions. The action was equal both qualitatively and quantitatively to that elicited by the same strength solution of *dl*- $\beta$ -phenylisopropylamine and greater than that produced by *l*-ephedrine. Although *l*-ephedrine did increase the tonus to about the same degree the individual contractions were not as completely suppressed. After the uterine strips were first treated with ergotoxine, *l*-*N*-ethylephedrine still produced a marked increase in tonus, whereas *l*-ephedrine had no effect. Typical results are presented in figure 5.

*Central Stimulation.* The degree of central stimulation produced by *l*-*N*-

ethylephedrine was determined and compared with that elicited by similar amounts of *l*-ephedrine and *dl*- $\beta$ -phenylisopropylamine. Central stimulation was tested by measuring the total activity of white rats by means of the method described by Schulte *et al.* (7) and also by Dille (8). Eight animals were used in each experiment. The temperature was kept constant at 28-29°C. and fresh air was circulated throughout the cabinet at all times. Male rats weighing from 200 to 250 grams were used. All of the compounds were given orally as a solution in a dosage of 50 mgm. The control animals received water by stomach tube and thus received the same mechanical treatment as the test animals. The results presented (figure 6) on an average, include 12 animals for each compound and 200 animals for the control values. In fig. 6 the abscissa gives the time in hours, the ordinate the average number of revolutions per hour. All tests were run for a period of 6 hours following the administration of the drug. It will be noted that the controls showed some increase in activity during the first hour. This may have been due to the handling of the animals. *L-N*-

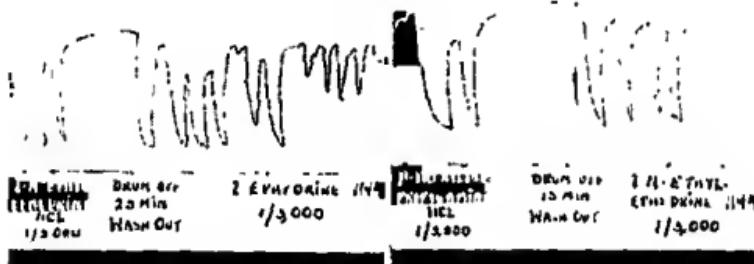


FIG. 5. COMPARATIVE EFFECT OF *L-N*-ETHYLEPHEDRINE, *L*-EPHEDRINE, AND *dl*- $\beta$ -PHENYLISOPROPYLAMINE ON THE ISOLATED UTERUS OF THE VIRGIN GUINEA PIG

Ethylephedrine produced little more activity than was present in the controls. The peak of activity was reached at the end of one hour. The animals receiving *l*-ephedrine reached their peak of activity in two hours and those receiving *dl*- $\beta$ -phenylisopropylamine reached the peak in three hours. At the end of the 6-hour period the activity of the control animals and those receiving the *L-N*-ethylephedrine was the same, whereas the activity of the animals in the other two groups was still above normal.

*Effect on the Pupil.* The mydriatic action of *L-N*-ethylephedrine following local application or intravenous administration in rabbits was only one-half to one-third as strong as that produced by similar amounts of *l*-ephedrine.

**DISCUSSION.** It is well known that the effects of secondary amines are qualitatively analogous to those of corresponding primary amines but that quantitative differences exist. These differences apparently are also carried over to tertiary amines, for Curtis (1) concluded that the conversion of a secondary to a tertiary amine did not remove all of the characteristic effects of the secondary amines. *L-N*-Ethylephedrine, a tertiary amine, differs from *l*-

ephedrine in that the hydrogen on the nitrogen atom has been replaced by an ethyl radical, and from *l*-*N*-methylephedrine in that it contains an additional carbon atom in one of the alkyl groups on the nitrogen. From the data given above it is apparent that qualitatively the action of all three of these compounds is very much the same but that there are quantitative differences.

Chen *et al.* (9) pointed out that an increase in the number of carbon atoms on the alpha carbon or on the nitrogen atom or a change from a secondary to a tertiary amine increases the toxicity. The results of our toxicity studies indicate that *l*-*N*-ethylephedrine is slightly more toxic acutely than *l*-ephedrine, though our chronic toxicity tests indicate that prolonged administration of *l*-*N*-ethylephedrine does not produce any pathological changes. Furthermore

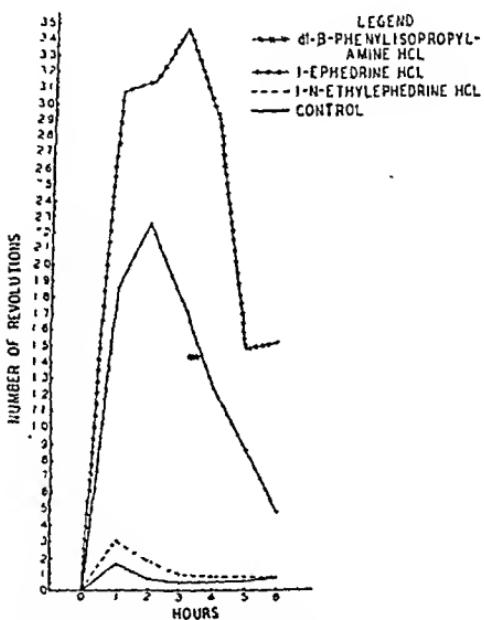


FIG. 6. COMPARATIVE CENTRAL STIMULANT ACTION OF *l*-*N*-ETHYLEPHEDRINE, *l*-EPHEDRINE, AND *dl*- $\beta$ -PHENYLISOPROPYLAMINE

these experiments gave an indication that there is no tolerance development with this compound since an M.L.D. still killed these animals after the daily administration of 25 mgm. for a period of four weeks.

In his preliminary studies on a number of tertiary amines related to *l*-ephedrine, Curtis (1) found that *l*-*N*-ethylephedrine produced a rise in blood pressure as great as, or greater than that elicited by similar amounts of *l*-*N*-methylephedrine. These results do not agree with those of the present investigation. The difference may have been due to the presence of some unaltered *l*-ephedrine in the sample employed by Curtis. In earlier experiments in this laboratory somewhat similar results were obtained but it was found that these samples contained small amounts of unreacted *l*-ephedrine.

Chen and his coworkers (9) pointed out that when a secondary amine is converted to a corresponding tertiary amine there is a decrease in the pressor effect. They found further that if the number of carbon atoms on the alkyl group on either the alpha carbon atom or the nitrogen was increased beyond two a depressor action became prominent. *l-N*-Ethylephedrine, a tertiary amine, definitely affects the blood pressure less than the secondary amine, *l*-ephedrine. It also exerts less pressor action than *l-N*-methylephedrine and this may be due to the fact that one of the alkyl groups on the nitrogen atom of *l-N*-ethylephedrine contains two carbon atoms.

Curtis (1) found that *l-N*-ethylephedrine and *l-N*-methylephedrine both dilate the bronchioles to approximately the same extent as *l*-ephedrine. Pak and Read (3) in some studies with *l-N*-methylephedrine found that the dilator effect of this compound was slightly less than that elicited by similar amounts of *l*-ephedrine. They also found that the onset of action was somewhat slower but the duration of action was of the same order. Thus the results of these authors as well as those of the present investigation indicate that the conversion of *l*-ephedrine to a corresponding tertiary amine does not alter significantly its bronchial dilator action. This action of *l-N*-ethylephedrine, like that of *l*-ephedrine, is mostly peripheral since it is present in pithed animals.

This compound appears to have a greater effect on uterine muscle than does *l*-ephedrine and it also has a greater spasmolytic action on the isolated intestinal strip. This latter action agrees very well with the observations of Hauschild (11) who found that the conversion of a secondary to a tertiary amine or loading the nitrogen atom increased the spasmolytic action.

The lack of central stimulant action of *l-N*-ethylephedrine fits in very well with the results of Schulte *et al.* (10). They found that the addition of a second methyl group to the amino group decreased this action. Hauschild (11) also observed that loading the nitrogen atom decreased the central stimulant action. In the present compound the addition of the ethyl group has apparently nullified the central stimulant action.

Thus *l-N*-ethylephedrine differs from *l*-ephedrine in that it is slightly more toxic, exerts much less pressor and central stimulant action, and elicits a somewhat greater spasmolytic action on the isolated intestine. Like *l*-ephedrine this compound is active following both oral and parenteral administration and both elicit approximately the same degree of bronchial dilatation in the experimental animal. Clinically *l-N*-ethylephedrine may prove useful in the treatment of certain types of bronchial asthma since it should produce fewer undesirable side-reactions.

#### SUMMARY

The toxicological and pharmacological actions of *l-N*-ethylephedrine have been described and compared with those of *l*-ephedrine.

The toxicity of this compound is of the same order as that of *l*-ephedrine.

The effect on the cardiovascular system is less than that elicited by similar amounts of *l*-ephedrine whereas the effect on the bronchioles is approximately the same.

*l*-*N*-Ethylephedrine appears to have a greater effect on the intestinal and uterine muscle than *l*-ephedrine.

This compound produces a minimum of central stimulation.

An attempt has been made to correlate the pharmacological actions of *l*-*N*-ethylephedrine with its chemical structure.

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# A STUDY OF SOME BETA-2, AND 4, PYRIDYLALKYLAMINES

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A series of beta-2, and 4, pyridylalkylamines described by Walter *et al.* (1) were originally investigated with the object of ascertaining their suspected sympathomimetic activity. Pressor activity might be anticipated for these derivatives as Hartung (2), in an excellent review on epinephrine and related compounds, concluded that "The aromatic portion of the molecule need not be a phenyl or a substituted phenyl group. Various naphthalene and heterocyclic derivatives also possess pressor activity."

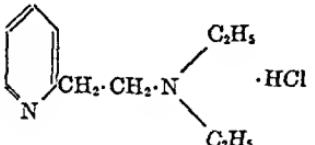
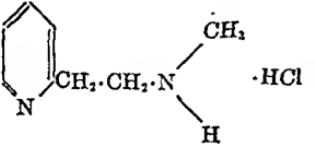
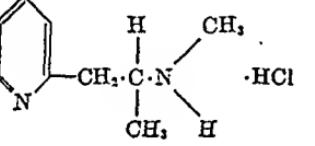
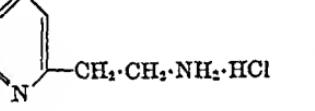
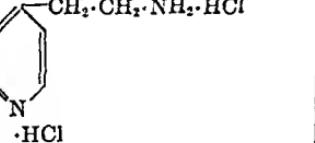
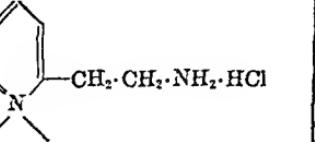
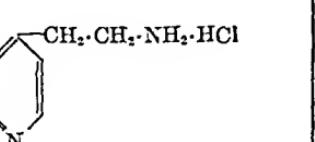
Preliminary studies on the effect of the beta-2 pyridylalkylamines on the blood pressure of anesthetized rabbits and cats revealed that they did not behave like epinephrine and related compounds but rather closely resembled histamine in action. Subsequent investigative efforts were directed to a study of the effect of these derivatives (a) on the circulatory and respiratory systems following intravenous administration in the anesthetized cat and rabbit and intraperitoneal administration in the guinea pig, (b) on isolated smooth muscle tissue and (c) intravenous toxicity in the rabbit. The chemical names, identification numbers and structural formulas of the compounds studied are listed in table 1.

**CIRCULATORY AND RESPIRATORY EFFECTS.** A series of experiments were conducted to determine the effect of these compounds on the blood pressure and respiration of the cat and rabbit, anesthetized by intraperitoneal injection of an aqueous solution of urethane (1 gram per kgm.). The usual technique was employed to record the arterial pressure while the respiratory movements were recorded from the trachea. All of the amines investigated with the exception of beta-4-pyridylethylamine dihydrochloride, beta-2, and 4, pyridylethylamine *N*-methocloride dihydrochlorides, produced a moderate to marked fall in blood pressure following intravenous administration to cats in doses of 3 to 8 mgm. In rabbits a pressor response was obtained when the compounds were administered by the same route. The depressor effect on the blood pressure of the cat was not altered when atropine sulfate was injected intravenously in an amount sufficient to inhibit vagal stimulation. Figure 1 illustrates the blood pressure effects obtained.

From fig. 1 it may be seen that relatively large doses of the 2-pyridylalkylamines were required to approximate the depressor effect of 12 micrograms per kgm. of histamine phosphate injected intravenously and in this respect the activity of these compounds differs considerably. The effects of the depressor amines on the respiratory movements of the anesthetized cat were but slight and appeared to be secondary to the changes in blood pressure.

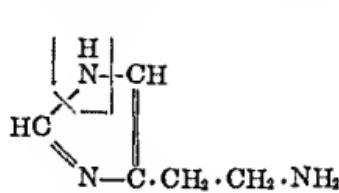
There was a definite but transitory rise in blood pressure following the administration of beta-4-pyridylethylamine dihydrochloride and when it was given

TABLE 1

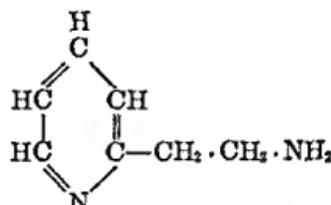
IDENTIFI- CATION NUMBER	FORMULA	CHEMICAL NAME
PT-8		$\beta$ (2-pyridyl) ethyldiethylamine Hydrochloride
PT-9		$\beta$ (2-pyridyl) ethylmethylamine Hydrochloride
PT-10		1, (2-pyridyl) 2, (methylaminopropane) Hydrochloride
PT-11		$\beta$ (2-pyridyl) ethylamine Hydrochloride
PT-20		$\beta$ (4-pyridyl) ethylamine Dihydrochloride
PT-47		$\beta$ (2-pyridyl) ethylaminemethochloride Hydrochloride
PT-48		$\beta$ (4-pyridyl) ethylaminemethochloride Hydrochloride

after ergotamine tartrate it produced a typical "epinephrine reversal" response. This amine, from its pressor response, acted like epinephrine hydrochloride but was far less active. Beta-2, and 4, pyridylethylamine-*N*-methoehloride hydrochlorides in like dosage did not influence the blood pressure and respiratory movements of the anesthetized cat.

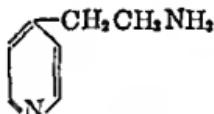
The difference in blood pressure response of the rabbit and cat brought about by the intravenous administration of PT-8, 9, 10 and 11 hydrochlorides appeared to be qualitatively indistinguishable from that produced by histamine. A comparison of the structural formula of beta-2-pyridylethylamine with that of histamine reveals an interesting and striking relationship.



Histamine (a)



Beta-2-pyridylethylamine (b)



Beta-4-pyridylethylamine (c)

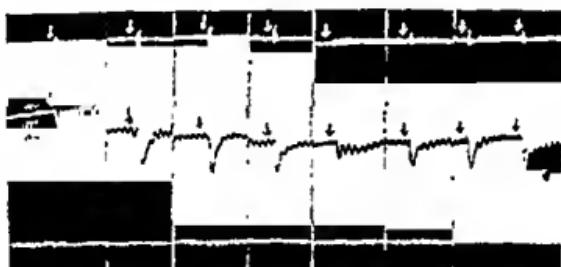


FIG. 1. CAT. URETHANE ANESTHESIA. DRUGS GIVEN INTRAVENOUSLY

From top down—Respiration, blood pressure, zero pressure line, time in 15 seconds. Left to Right—8.5 mgm. PT-8, 3 mgm. PT-11, 3 mgm. PT-9, 0.02 mgm. Histamine Phosphate. After 1 mgm. per kgm. Atropine Sulfate. 8.5 mgm. PT-8, 3 mgm. PT-9, 3 mgm. PT-11, 0.02 mgm. Histamine Phosphate.

An obvious similarity in structure exists between (a) and (b), for they differ only by replacement of an NH group by a HC=CH group, while (c) resembles an aromatic alkylamine and would be expected to have "epinephrine-like" activity.

A study was made to determine the comparative bronchio-spastic activity of the pyridylalkylamines in relation to histamine phosphate when injected intraperitoneally to guinea pigs. Three animals were employed for each compound and the doses were calculated on the basis of intravenous toxicity to rabbits.

Histamine phosphate was injected in a dose of 10 mgm. per kgm. producing the same type of death in each animal. Respiratory movements were slowed approximately eight minutes after injection and became very labored at thirteen minutes, followed by convulsions for about three minutes, with cessation of respiration at the end of eighteen minutes. Respiration ceased before the heart stopped beating. At cessation of respiration the thoracic cavity was exposed for examination. It was noted that the lungs were blanched and the heart, though beating feebly, was very cyanotic. Similar experiments were carried out with PT-8, 9, 10 and 11 using doses of 300, 40, 300 and 30 mgm. per kgm. respectively. All of the compounds exhibited the same characteristic response as histamine phosphate with the exception of PT-8 which failed to show the characteristic blanched lung. From these results it may be said that PT-9, 10 and 11 produce death by respiratory failure.

The similarity in the action of these compounds and histamine phosphate upon peripheral vasodilation was exhibited by intradermal injection in the guinea pig. PT-8, 9, 10 and 11 in doses of 1 mgm. and 0.01 mgm. histamine phosphate produced a well-defined and marked erythematous area around the site of injection.

An investigation of the effect of these compounds upon the circulation in the rabbit's ear was made on male white New Zealand strain rabbits which were lightly anesthetized with ether. The compound was injected into the right femoral vein and the effect noted. Groups of three rabbits were used for each test in which 50 and 75% of the maximum tolerated doses were injected. Histamine phosphate, PT-8, 9, 10 and 11 produced slight to medium dilation of the ears following injection of the lower dose whereas the higher produced definite dilation. No increase in salivary secretion was noted in any of the animals.

The effect of these derivatives upon the coronary circulation of the denervated isolated heart of the cat was studied following the technique described by Katz *et al.* (3). Two animals were employed for each determination. In this series of tests aqueous solutions were prepared from the crystalline pyridylalkylamine dihydrochlorides. When it was necessary to inject highly concentrated solutions, as in the case of PT-10, neutralization of the dihydrochloride to the mono-hydrochloride was required to avoid a spurious vaso-constrictor effect produced by the acidity of the solution. Aqueous solutions thus prepared had a pH of 6. Histamine phosphate was used as a control and the amounts of the amine salts injected were, in general, proportionate to their intravenous lethal doses in the rabbit. The volume of the solution injected into the rubber tubing leading to the aorta was not less than 0.5 cc. nor more than 0.75 cc. The solution was administered instantaneously, whereas Katz *et al.* gave their injections at a constant rate over a period of one minute or less. It was found by experiment that the more rapid rate of injection of the volumes indicated above did not significantly alter the response.

From the results obtained and partially illustrated in table 2 it may be concluded that these compounds either dilate the coronaries or produce no change in their function and do not produce vaso-constriction.

**ISOLATED TISSUE.** The depressor behavior of the beta-2-pyridylalkylamines led us to study their action on isolated strips of smooth muscle, such as the uterus and intestine of the guinea pig and rabbit. With the exception of beta-2 and 4 pyridylalkylamine *N*-methoehlorides, which were inactive, all of the compounds exhibited a potent stimulating action on isolated strips of guinea pig uterus (fig. 2) and intestine. Table 3 shows the concentrations of the various

TABLE 2

*Effect of amines upon rate of flow through the coronaries of isolated cat heart*

COMPOUND	QUANTITY OF AMINE INTRODUCED	AVERAGE RATE OF FLOW OF CONTROL FOR 5 MINUTES	AVERAGE RATE OF FLOW AFTER AMINE			
			1-5 minutes	5-10 minutes	10-15 minutes	15-20 minutes
PT-8	10	12.8	16.1	15.2		
PT-9	2	33.1	30.1	29.5	30.2	
PT-10	75	24.5	31.7	33.5	30.7	
PT-11	2	41.9	43.3	43.8	44.9	43.9
Histamine phosphate	1	41.9	53.4	56.9	46.9	42.4



FIG. 2. GUINEA PIG UTERUS, ISOLATED STRIPS. 100 CC. CHAMBER

Left to Right—At first ↑ each tracing,  $8 \times 10^{-6}$  PT-8,  $6 \times 10^{-6}$  PT-9,  $1.5 \times 10^{-5}$  PT-10,  $3 \times 10^{-6}$  PT-11 and  $2 \times 10^{-7}$  Histamine Phosphate.  
Second ↑ in each tracing indicates Wash.  
Time in 15 seconds.

active agents, in micrograms per cc., required to produce near-maximal contractions of the muscle. It will be noted that the activity of PT-9, 10 and 11 approaches that of histamine phosphate, while PT-8 was definitely inferior. Too much emphasis should not be placed on the quantitative aspects of these values.

While all of the beta-2-pyridylalkylamines and histamine phosphate exerted a stimulant effect on rabbit uterus and intestine (figs. 3 and 4), these tissues were far less sensitive, for doses of the compounds 5 to 27 times the size of those used in the guinea pig work were required to produce submaximal contractions.

Ergotoxine ethanesulfonate, in the case of rabbit uterus, and atropine sulfate in the case of rabbit intestine, in concentrations of  $1 \times 10^{-6}$  failed to inhibit the stimulant action of the amines. Following atropine sulfate 1:1,000,000, PT-10, in a concentration of  $1.6 \times 10^{-4}$  did not stimulate the isolated intestine, as shown in figure 4. When a greater concentration of PT-10 was in contact with the tissue a stimulant effect was observed and this would appear to indicate that the action of this compound is primarily through the parasympathetics.

A study of the effect of the pyridylalkylamines upon the isolated white rat uterus was made for the purpose of determining the activity of these com-

TABLE 3

*Concentrations of the amines producing near maximal contractions in guinea pig uterus and intestine (100 cc. chamber used)*

COMPOUND	CONCENTRATION OF AMINE BASE	
	Guinea pig uterus μgm./cc.	Guinea pig intestine μgm./cc.
PT-8	80	6
PT-9	6	2-3
PT-10	5	1
PT-11	3	1
Histamine phosphate	0.2	0.2

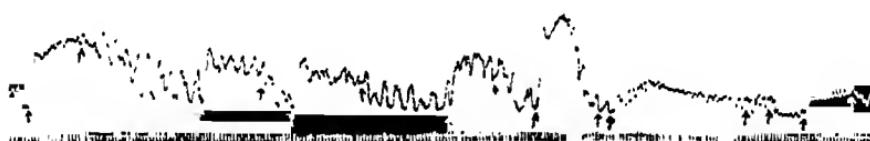


FIG. 3. ISOLATED RABBIT UTERUS. 100 cc. CHAMBER

Left to Right—At ↑  $4 \times 10^{-4}$  PT-8, Wash,  $8 \times 10^{-5}$  PT-9, Wash,  $8 \times 10^{-5}$  PT-10, Wash,  $8 \times 10^{-5}$  PT-11, Wash,  $1 \times 10^{-6}$  Ergotoxine Ethanesulfonate, End of 10 minute contact with tissue,  $1 \times 10^{-7}$  Epinephrine HCl,  $4 \times 10^{-4}$  PT-8, Wash.

Time in 15 Seconds.

pounds in comparison to that of histamine phosphate. The latter compound in a concentration of  $3 \times 10^{-6}$  produced almost complete relaxation of the tissue. PT-8 in a concentration of  $8 \times 10^{-5}$  produced definite stimulation of the tissue, whereas PT-9, 10 and 11 were ineffective in a concentration of  $2 \times 10^{-5}$ . Thus, the pyridylalkylamines do not resemble histamine in their action upon isolated rat uterus.

INTRAVENOUS TOXICITY. The intravenous toxicities of the beta-2-pyridylalkylamines and histamine phosphate were determined in white New Zealand strain rabbits. The data obtained are shown in detail in table 4. The LD-50

and the standard error were calculated according to the method of Litchfield and Fertig (4).

From our observations it would appear that all of these compounds are definitely less toxic than histamine. Most surprising to us, however, was the differ-

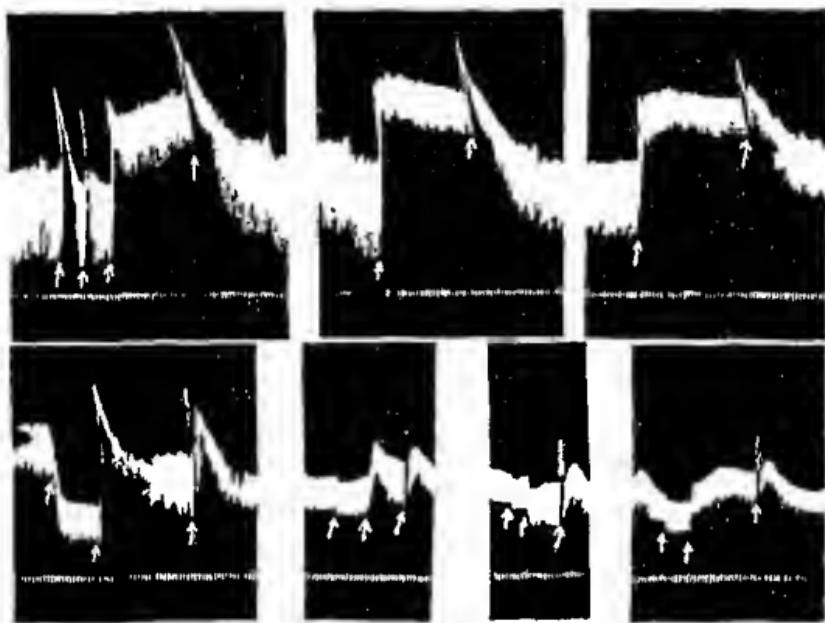


FIG. 4. ISOLATED RABBIT INTESTINE. 100 cc. CHAMBER  
 $10^{-4}$  PT-8, Wash,  $1.6 \times 10^{-4}$  PT-9, Wash,  $1.6 \times 10^{-4}$  PT-10,  
 $1 \times 10^{-4}$  Atropine Sulfate,  $4 \times 10^{-4}$  PT-8, Wash,  $1 \times 10^{-4}$   
, Wash,  $1 \times 10^{-4}$  Atropine Sulfate,  $1.6 \times 10^{-4}$  PT-10, Wash,  
 $10^{-4}$  PT-11, Wash.

Time in 15 Seconds

TABLE 4

COMPOUND	CALCULATED LD <sub>50</sub> AMINE BASE mgm./kgm.	S.E.	plf SOLUTION
PT-8	47.9	$\pm 3.4$	5.3
PT-9	5.1	$\pm 0.6$	4.7
PT-10	75.0	$\pm 3.6$	5.9
PT-11	3.8	$\pm 0.8$	5.8
Histamine phosphate	1.0	$\pm 0.02$	4.1

ence in toxicity between PT-9 and PT-10 which vary in chemical constitution only by the substitution of a methyl group for hydrogen on the carbon atom alpha to the methylamino group. A careful characterization of PT-10 confirmed the structure and purity so the lower toxicity must be ascribed to a specific structural difference.

Lethal doses of all compounds as well as histamine phosphate produced a bronchio-spastic death, the animals exhibiting cyanosis. The compounds when administered intravenously exhibited a chain of responses which may be described as follows. Approximately one minute after the intravenous injection of a lethal dose the animal developed a swaying motion and respiratory movements were labored. The intensity of this action increased until convulsions appeared accompanied by intermittent tremors of skeletal muscles. Death occurred in the majority of cases within a period of five minutes. Animals surviving after doses equivalent to LD-50—80 exhibited light intermittent convulsive movements with practically no tremors of the skeletal muscles. This condition persisted until the animal was able to regain movement. The average time of recovery was approximately 20 minutes.

PT-8 was found to produce violent tremors of skeletal muscles in all animals injected. These tremors developed into mild or strong convulsions depending upon the dose employed but at the maximum tolerated dose convulsions were observed in only one animal for a period of about two minutes.

TABLE 5

*Approximate maximum tolerated doses of amine bases administered intravenously to the rabbit*

COMPOUND	M. T. D. mgm./kgm.
PT-8	37.0
PT-9	2.0
PT-10	61.0
PT-11	2.0
Histamine phosphate	0.6

PT-9 and 11 when injected intravenously exhibited a behavior which closely paralleled that observed with histamine phosphate.

Delayed deaths were observed with PT-10. At a dose approximating the LD-50 two animals died 26 to 41 hours following injection while another died in 7 days. From the time of injection until death these animals exhibited severe respiratory embarrassment and suffered from diarrhea. At the approximate LD-20 dose one animal died one to fifteen hours following injection while four animals receiving the same dose showed no effects.

The approximate values for the maximum tolerated doses of histamine and the new derivatives are shown in table 5.

#### SUMMARY AND CONCLUSIONS

Histamine phosphate appeared to be at least one hundred times more active than the 2-pyridylalkylamine hydrochlorides in depressing the blood pressure when administered intravenously to the anesthetized cat. The variations in respiratory movements observed are believed to have been secondary to the blood pressure changes. The pyridylethylamine *N*-methochloride hydrochlorides were without effect on the blood pressure. Beta-4-pyridylethylamine dihydro-

chloride produced a pressor response similar to, but much weaker than epinephrine. The fall in blood pressure produced by the 2-pyridylalkylamines is believed due to peripheral dilation of blood vessels since we observed definite vasodilation of the vessels in the rabbit ear of the same side in which the cervical sympathetic was intact.

A study of the 2-pyridylalkylamines, resembling histamine in action, upon guinea pig revealed that death was produced by respiratory failure.

From the results obtained with the isolated denervated cat heart the 2-pyridylalkylamines either dilate the coronaries or produce no change in their function.

The activity of PT-9, 10 and 11 upon isolated guinea pig uterus and intestine approaches that of histamine phosphate, while PT-8 was definitely inferior. Rabbit uterus and intestine were stimulated by all of the 2-pyridylalkylamines, however PT-8 appeared to differ in its action on the intestine. None of the pyridylalkylamines behaved like histamine in their action upon the isolated white rat uterus.

It has been shown that all of the 2-pyridylalkylamines were definitely less toxic than histamine when administered intravenously to the rabbit.

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# THE EFFECT OF VARIOUS PHARMACOLOGICAL AGENTS ON THE MORPHOGENETIC ACTIONS OF ESTRADIOL

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It is well known that close pharmacological correlations exist between the various steroid hormones. In some respects they may antagonize each other, while in other respects they act synergistically. The knowledge of such synergisms and antagonisms is of great importance in therapeutics as it enables the clinician to accentuate a desired action and at the same time prevent harmful side effects by treatment with suitable hormone combinations.

In a preliminary study of this type (1) it has been shown that the testis atrophy produced by large doses of a folliculoid compound such as estradiol can be counteracted by simultaneous administration of progesterone or testosterone, while other effects of estradiol, for instance its ability to cause involution of the thymus, are actually accentuated by concurrent treatment with these steroids. The object of the present investigation was to make a systematic study of the effect of a great many pharmacological agents—mainly steroids—on the morphological changes induced by estradiol treatment in the pituitary, adrenal, thymus, testis, accessory sex organs and kidneys of the rat.

**METHODS.** Twenty-five groups of male albino rats were used in this experimental series. Each group consisted of six animals having an average weight of 154-159 g. at the beginning of the experiment. One group served as untreated controls, while all other groups received 150 micrograms of estradiol subcutaneously twice daily in 0.1 cc. of peanut oil, together with subcutaneous injections of various other compounds which were likewise administered bidayly in 0.1 cc. of peanut oil. This treatment was continued for 14 days. On the 15th day all animals were sacrificed and their organs weighed on an analytical balance after fixation in "Susa" solution.

The results of our experiments are summarized in table 1 which is almost self-explanatory. The full systematic name of each compound is used in order to avoid confusion, but in all those cases in which the substance is generally referred to by a common name the latter is also given (in italics). The melting point of the sample which was available to us for these experiments was determined in our laboratory and is given in the table as an indication of the degree of purity of the preparation used. These melting points will also facilitate the identification of the compound whenever there is doubt about several possible isomerids. In the case of *d,l*-alpha-tocopherol acetate, which is fluid at room temperature, as well as in the case of the brain extract and prolactin, which were not pure crystalline products, a melting point obviously cannot be given. The brain extract consisted of the petroleum-ether-soluble lipids of three ox brains, and was used in order to determine whether the lipids naturally occurring in central nervous system tissue would influence the morphogenetic actions to estradiol. The prolactin preparation was a technical product containing 12.5 I.U.

per mg. It was assayed in this connection mainly because of its recently discovered direct trophic effect on the corpus luteum cells of the ovary. The actions of estradiol are not influenced by such inert steroids as cholesterol or stigmasterol. Hence the groups receiving estradiol in combination with these sterols are ideal controls showing the unmodified actions of estradiol.

It will be noted that although estradiol was always administered in the same dose, the other compounds were given in doses of 10 mg. per day in groups 2-18 and at a 2 mg. daily dose level in groups 19-25. Only those compounds were tested at a low dose level which proved definitely effective in preventing testis atrophy at the dose of 10 mg. per day. Since the main object of our work was to study the ability of these substances to prevent testis atrophy after estradiol treatment both in the 10 mg. and in the 2 mg. dosage group, the compounds are enumerated according to descending testis-protecting potency. Average body and organ weights are given in each case but the maximum range (in parenthesis) is also mentioned.

The *body weight*, which is known to diminish under the influence of estradiol overdosage, showed a definite decline during the experimental period in most of those groups in which estradiol was administered in combination with hormonally inactive compounds such as vitamin E (group 9), cholestenone (group 10), cholesterol (group 13) or stigmasterol (group 14). In other cases it remained stationary or revealed only an insignificant rise. Among all compounds examined androstanediol (groups 3 and 22) induced the best weight increase both in the 10 mg. and in the 2 mg. dose. It will be recalled that, since McEuen *et al.* (2) first showed that chronic treatment with testosterone markedly stimulates somatic growth, both in the male and in the female rat, numerous investigators confirmed this action of testosterone and other testoid compounds in man, as well as in experimental animals (3, 4, 5). It is perhaps not without interest to note that androstanediol is even more potent in this respect than testosterone since in short-term experiments, such as the present, the latter proved practically inactive while androstanediol exhibited a definite effect. Since the "male hormone" or testoid activity of androstanediol (judged by assays on the capon or the castrate rat) is far inferior to that of testosterone, the body growth-stimulating action of steroids appears to be relatively independent of their testoid potency. This may prove to be of clinical interest as it indicates that androstanediol is preferable to testosterone if maximum body growth stimulation is desired without too much "masculinization." However, none of the compounds examined restored the growth rate to anything like the normal level.

The weight of the *pituitary* increases under the influence of estradiol and this increase remains quite evident in most groups receiving this hormone in combination with other compounds. Only methyl testosterone (group 4) and testosterone (group 5), at the 10 mg. dosage level, are able to inhibit this reaction completely. This is rather noteworthy since Clarke *et al.* (6) demonstrated that all hormonally active steroids exert a definite morphological action on the pituitary of gonadectomized male or female rats inasmuch as they prevent the development of castration changes.

The *adrenal* weight is known to increase under the influence of pure crystalline

TABLE I  
The effect of various pharmacological agents on the morphogenetic actions of estradiol

TREATMENT	INITIAL BODY WEIGHT IN G.	FINAL BODY WEIGHT IN G.	ADRENALIN IN MG.	THYROID IN MG.	TESTIS IN G.	SEMINAL VESICLES IN MG.	VENTRAL PROSTATE IN MG.	MIDDLE PROSTATE IN MG.	PREPUTIAL GLAND IN MG.	EPIDIDYMIS IN MG.	KIDNEY IN G.	COAGULATING GLAND IN MG.	
1. Not injected	159 (148-173)	206 (180-240)	8 (7-8.6)	26 (21-36)	225 (176-353)	2,485 (2,150-2,817)	436 (357-529)	202 (159-282)	157 (125-212)	623 (613-724)	61 (37-81)	26 (33-96)	1,709 (1,570-2,306)
2. $\Delta^4$ -Androstene-3 $\beta$ -ol-17-one Dihydro-iso-androsterone 10 mg.	137 (140-175)	154 (160-180)	8 (7-9)	31 (26-38)	34 (2,050-3,149)	2,460 (074-1,063)	869 (379-416)	395 (190-230)	231 (074-920)	749 (54-151)	94 (135-207)	165 (135-207)	1,708 (1,677-1,814)
3. $\Delta^4$ -Androstene-3 $\beta$ -ol-17 $\alpha$ -diol Androsterone 10 mg.	162-163 (145-170)	159 (160-195)	172 (8-14)	39 (33-47)	70 (16-116)	2,375 (2,160-2,555)	765 (632-960)	286 (241-382)	224 (220-227)	678 (57-782)	102 (72-145)	120 (61-163)	1,663 (1,398-1,977)
4. 17-methyl- $\Delta^4$ -androstene-3 $\beta$ -one- 17 $\alpha$ -ol Methyl testosterone 10 mg.	153 (140-175)	156 (135-195)	7 (7-9)	42 (32-54)	23 (15-36)	2,215 (1,835-2,395)	1,256 (1,054-1,572)	372 (345-427)	314 (245-373)	659 (151-759)	77 (67-85)	217 (137-233)	1,870 (1,642-2,063)
5. $\Delta^4$ -Androstene-3 $\beta$ -one-17 $\alpha$ -ol Testosterone 10 mg.	154 (145-177)	160 (145-175)	7 (6-8.5)	35 (31-39)	29 (19-45)	2,199 (1,386-2,562)	1,428 (1,292-1,772)	448 (305-331)	358 (287-437)	597 (508-791)	72 (53-92)	220 (165-288)	1,859 (1,659-2,157)
6. 17 $\alpha$ -ethyl- $\Delta^4$ -Androstene-3 $\beta$ -ol-17 $\alpha$ - one $\Delta^4$ -Pregnenolone 10 mg.	183 (145-170)	156 (130-165)	143 (9-13)	11 (33-45)	38 (50-150)	2,176 (1,755-2,645)	104 (93-126)	116 (97-132)	116 (68-144)	429 (297-569)	52 (43-72)	28 (16-48)	1,526 (1,387-1,541)
7. 17 $\alpha$ -ethyl- $\Delta^4$ -Androstene-3 $\beta$ -ol- dione Progesterone 10 mg.	128 (150-176)	156 (145-180)	11.6 (10-13)	31 (26-38)	65 (31-115)	1,892 (1,603-2,119)	125 (96-159)	108 (77-139)	123 (77-132)	399 (313-510)	62 (29-88)	33 (26-45)	1,503 (1,338-1,759)
8. Petroleum ether soluble lipids of 3 ox brains 10 mg.	150 (140-180)	168 (135-180)	14 (11-16)	41 (32-47)	109 (67-150)	1,564 (0,970-2,308)	50 (32-68)	39 (13-61)	51 (22-73)	258 (147-417)	47 (38-70)	14 (7-18)	1,639 (1,369-1,835)
9. D,L- $\alpha$ -tocopherol acetate Vitamin E 10 mg.	157 (145-175)	147 (125-150)	11 (8-13)	43 (38-50)	77 (39-138)	1,560 (1,172-2,107)	57 (45-67)	44 (34-84)	49 (30-72)	239 (161-362)	43 (33-57)	17 (12-24)	1,476 (1,354-1,762)
10. 17 $\alpha$ -isooctyl- $\Delta^4$ -Androstene-3 $\beta$ -one Cholestanone 10 mg.	80.5 (140-175)	157 (135-175)	151 (10-12)	10 (35-53)	96 (70-124)	1,550 (1,020-2,131)	61 (47-71)	42 (28-62)	46 (30-50)	245 (157-331)	38 (26-58)	17 (14-20)	1,029 (1,454-1,841)
11. Acetone soluble lipids of 3 ox hypothalamus 10 mg.	158 (135-175)	157 (135-185)	10 (6-12)	45 (35-58)	99 (36-137)	1,500 (1,032-1,677)	61 (41-81)	54 (29-116)	58 (30-89)	234 (136-341)	33 (19-51)	18 (14-23)	1,692 (1,355-1,767)

<sup>a</sup> *Produced no increase in weight.*

<sup>b</sup> *Produced increase in weight.*

<sup>c</sup> *Produced increase in weight.*

<sup>d</sup> *Produced increase in weight.*

<sup>e</sup> *Produced increase in weight.*

<sup>f</sup> *Produced increase in weight.*

13	Progesterone 10 mg.	(145-175) (158-175) (13-17)	(10-46)	(119-204) (10-36)	(118-201) (22-6)	166 (20-30)	166 (25-36)	46 (10-24)	46 (118-201) (26-71)	16 (1-24)	1,554 (1,300-1,713)
14	17-hydroxy- $\Delta^4$ -androstene-3 $\beta$ -ol- Chloroform 10 mg.	149 (145-177) (152-160) (9-16)	158 (145-177) (152-160) (9-16)	145 (145-177) (152-160) (8-11)	12 (27-35)	39 (10-133)	41 (10-133)	1,256 (45-63)	55 (19-38)	20 (34-10)	32 (145-275) (17-49)
15	17-styryl- $\Delta^4$ -androstene-3 $\beta$ -ol- diol- $\alpha$ -D-acetate	168-169 (143-177) (150-185)	155 (140-180) (145-180)	151 (140-180) (145-180)	9 (8-11)	33 (29-53)	97 (30-124)	1,152 (43-65)	49 (24-51)	30 (21-36)	35 (100-555) (25-48)
16	Progesterone 10 mg.	153-158 (140-180) (145-180)	156 (140-180) (145-180)	163 (140-180) (145-180)	11 (8-11)	44 (32-53)	109 (29-133)	1,141 (41-72)	61 (14-76)	39 (23-59)	32 (144-352) (27-47)
17	17-ethynyl- $\Delta^4$ -androstene-3 $\beta$ -ol- diol- $\alpha$ -D-acetate	205-208 (140-175) (145-170)	139 (140-170) (145-170)	123 (140-175) (145-175)	10 (8-11)	43 (31-50)	65 (15-97)	1,057 (41-70)	64 (58-78)	201 (47-70)	35 (144-352) (27-47)
18	17-ethynyl- $\Delta^4$ -androstene-3 $\beta$ -ol- diol- $\alpha$ -D-acetate	152 (140-170) (145-165)	155 (140-170) (145-165)	144 (140-170) (145-165)	9 (7-11)	48 (32-52)	89 (68-132)	1,057 (180-317)	63 (14-76)	129 (13-43)	35 (144-352) (27-47)
19	17-ethynyl- $\Delta^4$ -androstene-3 $\beta$ -ol- diol- $\alpha$ -D-acetate	166 (140-180) (145-180)	161 (140-180) (145-180)	161 (140-180) (145-180)	10 (9-11)	28 (35-41)	101 (76-133)	2,257 (2,068-2,427)	27 (73-84)	125 (14-51)	33 (277-423) (31-43)
20	17-ethynyl- $\Delta^4$ -androstene-3 $\beta$ -ol- diol- $\alpha$ -D-acetate 2 mg.	137 (145-175) (152-160)	156 (145-175) (152-160)	103 (145-175) (152-160)	10 (8-13)	40 (27-51)	121 (84-135)	2,104 (1,817-2,473)	197 (138-273)	132 (95-160)	67 (54-127) (374-545) (44-96)
21	Androstan-3 $\beta$ -ol- $\alpha$ -D-acetate	177-178 (140-175) (145-185)	157 (140-175) (145-185)	169 (140-175) (145-185)	12 (10-16)	38 (30-52)	98 (16-147)	2,045 (1,053-2,299)	283 (175-393)	193 (109-255)	67 (160-340) (300-623) (37-109)
22	Androstan-3 $\beta$ -ol- $\alpha$ -D-acetate 2 mg.	162-163 (140-180) (145-185)	154 (140-180) (145-185)	165 (140-180) (145-185)	8 (5-10)	40 (32-56)	78 (33-136)	1,943 (1,334-2,581)	452 (336-614)	106 (135-311)	65 (130-229) (337-778) (76-114)
23	17-methyl- $\Delta^4$ -androstene-3 $\beta$ -one- 7 $\alpha$ -ol	153 (130-170) (140-160)	150 (130-170) (140-160)	157 (130-170) (140-160)	5,5 (5-12)	35 (31-43)	60 (32-83)	1,913 (1,755-2,009)	815 (723-1980)	229 (221-323) (1143-225) (569-653)	75 (144-98) (112-182) (1,417-1,639)
24	17-ethynyl- $\Delta^4$ -androstene-3 $\beta$ -one- 7 $\alpha$ -ol	154 (138-180) (140-170)	159 (138-180) (140-170)	161 (138-180) (140-170)	13 (11-17)	36 (32-47)	67 (31-91)	1,825 (1,347-2,358)	932 (944-1940)	229 (224-312) (220-235) (416-704) (46-81)	67 (115-174) (112-182) (1,417-1,639)
25	17-ethynyl- $\Delta^4$ -androstene-3 $\beta$ -ol- diol Progesterone 2 mg.	125 (140-180) (145-185)	156 (140-180) (145-185)	143 (140-180) (145-185)	8 (7-9)	39 (33-45)	40 (14-79)	1,876 (1,233-2,030)	70 (55-104)	81 (37-119)	349 (45-133) (261-454) (31-55)

folliculoid compounds (7). This increase can be inhibited by desoxycorticosterone acetate, progesterone or testosterone (1, 8). The present experimental investigation confirms these facts but shows that comparatively high doses (10 mg. per day) are required to produce a definite inhibition. At this dosage level dehydro-*iso*-androsterone (group 2) also appears to show some effect, but desoxycorticosterone acetate (group 18) proves far superior to all other compounds investigated.

The *thymus* involution caused by estradiol is perhaps somewhat inhibited by pregnanediol (group 12) but the great individual variation in thymus weight makes this finding of doubtful value. The other compounds investigated were inert, while methyl testosterone and testosterone actually increased this effect of estradiol.

The *testis* atrophy caused by estradiol is most actively inhibited by  $\Delta^5$ -pregnenolone (group 19) in the low dosage series, while at the 10 mg. dose level several of the testoid compounds are somewhat more active. This is of particular interest since in castrate rats  $\Delta^5$ -pregnenolone is practically inactive as a testoid compound whence one may conclude that the gonad-protecting ability of steroids is independent of their testoid potency. It had previously been noted (9) that there is no close correlation between the gonad protecting activity of a steroid and its ability to maintain the accessory sex organs of the castrate male. Yet all gonad protecting substances so far observed proved to possess some measure of testoid activity and *vice versa*. The present experimental series revealed a testoid compound (ethinyl testosterone) which is devoid of gonad-protecting action and an active gonadotropic steroid ( $\Delta^5$ -pregnene- $\beta$ -ol-20-one) which proved inert as a testoid, when assayed on castrate rats. It appears justified to conclude, therefore, that this type of gonadotropic action is entirely independent of testoid activity.

It will be seen that at low dosage levels  $\Delta^5$ -pregnenolone is even more gonadotropic than testosterone, methyl testosterone, androstanediol or dehydro-*iso*-androsterone, while at the 10 mg. dosage level  $\Delta^5$ -pregnenolone is less effective than the above compounds. The explanation of this is probably given by the observation of Selye and Albert (17) who found that at low dosage levels all these testoids cause some degree of testis involution by themselves, while  $\Delta^5$ -pregnenolone does not decrease the testis size at any dose level. It will also be recalled that small doses of testosterone cause a testis atrophy which is not seen at high dose levels (11). This fact could best be explained by the assumption that comparatively low doses of testoids inhibit the gonadotropic hormone secretion of the pituitary and thus cause a secondary testis involution which, in the case of high doses, is over-compensated by the direct testis-stimulating effect of these compounds. The present findings are in accord with the previously reported observation (12) that  $\Delta^5$ -pregnenolone causes no testis atrophy at any dose level and is highly effective in protecting the gonads against atrophy produced by various means. This implies that it can safely be employed as a testis protecting drug without running the risk of causing testis damage at any dosage

level. Desoxycorticosterone acetate caused marked testis involution in itself when given at high dose levels (13). As was to be expected it aggravated the testis weight loss caused by estradiol.

The *accessory sex organs* undergo atrophy under the influence of estradiol treatment since their maintenance is dependent upon the endocrine function of the testis which is greatly deranged, if not completely suppressed by folliculoid compounds. Perusal of our table indicates that all hormonally inactive compounds (groups 8-14) failed to prevent this atrophy. Among hormonally active substances acetoxy pregnenolone (group 15) and desoxycorticosterone acetate (group 18) proved entirely inert in this respect while all other steroids inhibited the involution of the accessory sex glands more or less markedly and, as was to be expected, the active testoids actually caused them to hypertrophy. It is particularly noteworthy that  $\Delta^4$ -pregnenolone (group 6) and progesterone (group 7) which proved quite inactive in causing seminal vesicle enlargement in immature castrate rats (17, 14) elicited a marked hypertrophy in the present series. This apparent contradiction may be due to the circumstance that the above steroids are inactive in castrates because they have to be activated in some manner within the testis tissue. Another possible explanation, however, is that the animals of the present series proved highly sensitive to the above steroids merely because they were of post-pubertal age. It has been shown (10) that at puberty there is a sudden increase in the sensitivity of castrates to certain testoids while other testoid compounds are approximately equally effective in pre- and post-pubertal rats. Experiments are now under way to determine which of these two explanations is the correct one. Be this as it may, the fact remains that  $\Delta^4$ -pregnenolone and progesterone (groups 6 and 7) are markedly effective in stimulating the accessory sex organs under the conditions prevailing in these experiments, while this activity is destroyed by substitution of a hydrogen at C<sub>21</sub> by an acetoxy group which transforms these compounds into acetoxy-pregnenolone and desoxycorticosterone acetate (groups 15 and 18) respectively. It should also be emphasized that androstenediol is more potent than any other compound so far examined in causing hypertrophy of the preputial glands although it is less active in causing enlargement of seminal vesicles, prostates, epididymis and coagulating glands than the active testoids which we investigated, namely testosterone and methyl testosterone (groups 4, 5, 23 and 24). It appears that certain testoid compounds may preponderantly stimulate one or the other of the male accessory organs. A more generally known example of this is the fact that testosterone enlarges the seminal vesicles proportionally more than the prostate, while androsterone stimulates the latter organ more than the former.

The *kidney* enlarging or "renotropic" action of steroids has been extensively studied by Selye (15, 16) in intact, spayed and hypophysectomized animals. It has also been demonstrated (1) that estradiol causes kidney involution. In confirmation of previous findings it will be seen that the kidneys of animals receiving estradiol in combination with steroids devoid of renotropic action, show

a subnormal average weight and that the best renotropic effect is exhibited by the most active testoids such as testosterone and methyl testosterone (groups 4, 5, 23 and 24).

#### SUMMARY

The effect of various pharmacological agents (mainly steroids) on the morphological changes induced by estradiol has been studied in the rat. The findings which are given in a table are not suitable for brief presentation in the form of a summary but the following salient facts may be mentioned:

Among all compounds examined androstanediol proved most potent in preventing the body weight loss caused by estradiol. It also proved to have the greatest preputial gland stimulating effect although its testoid action—judged by the ability of the compound to enlarge the seminal vesicles, prostates and coagulating glands—is much inferior to that of other steroids in our series (methyl testosterone, testosterone).

Few steroids are able to prevent the pituitary and adrenal hypertrophy elicited by estradiol overdosage. The most clear-cut inhibition of the hypophyseal hypertrophy was obtained with high doses of testosterone and methyl testosterone. The adrenal weight increase was not only prevented, but actually reversed by desoxycorticosterone acetate.

The thymus involution caused by estradiol is accentuated by most hormonally active steroids.

Definite proof is furnished that the ability of steroids to protect the testis against the atrophy caused by estradiol is entirely independent of their "male hormone" or testoid activity. Ethinyl testosterone, though a potent testoid, is not gonad-protecting while the reverse is true of pregnenolone.

The decrease in kidney weight caused by estradiol is effectively prevented only by highly active testoids such as testosterone and methyl testosterone.

On the basis of examples furnished by the present experimental series it is emphasized that by the use of suitable steroid hormone combinations it is possible to accentuate certain desired effects and simultaneously inhibit some of the undesirable side effects of hormones thus increasing the pharmacological specificity of their action.

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# BRONCHIAL ANTISPASMODIC ACTIONS OF THEOPHYLLINE DERIVATIVES, INCLUDING EFFECTS OF CONTINUED ADMINISTRATION

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In recent years, aminophyllin (theophylline ethylenediamine) has been used extensively in the treatment of intractable bronchial asthma, with almost uniformly favorable results (1, 2, 3). This use was based on the empirical observation that patients receiving the drug for cardiac depression experienced definite relief from their dyspnea. Such relief could have been the result of improved cardiac efficiency or of bronchodilatation due to direct depression of smooth muscle in the bronchi. Depression of smooth muscle by theophylline is known for blood vessels (4, 5) including the coronaries, dilatation of which is indirectly responsible for cardiac stimulation. Experimentally, however, the bronchial antispasmodic action of theophylline derivatives has not been demonstrated on bronchi *in situ*, and only to a limited extent on excised bronchi. Young and Gilbert (6), using isolated bronchial rings of rabbits according to Sollmann and Gilbert's method (7), found that the direct application of aminophyllin overcame the bronchoconstriction of histamine. Gilbert and Goldman (8) showed the same for isolated bronchial rings of young dogs, the required concentrations of aminophyllin being high, i.e., 1:2000 to 1:1000. Aminophyllin diminished the severity of the symptoms and the mortality from histamine shock in guinea pigs, according to Young and Gilbert (6). None of these reports indicates the possible nature of the antispasmodic action of aminophyllin, as to whether this is general or limited to histamine spasm, whether specific for aminophyllin or produced by other theophylline derivatives, and whether ethylenediamine, a component of the aminophyllin complex, has a share in the effect. Quantitative differences have been claimed by Le Roy and Speer (9) in the actions of theophylline derivatives on the coronary vessels. Although the vasodilator action appeared to be derived from the methylxanthine, the combination with other chemical substances apparently increased or decreased it.

In view of the practical importance of the problem and the scanty attention which it has received pharmacologically, I have investigated the comparative actions of two theophylline derivatives and epinephrine on the bronchi in isolated and intact lungs. The two theophylline derivatives were aminophyllin<sup>2</sup> (theo-

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<sup>2</sup> These names are used throughout for convenience and brevity instead of the unwieldy chemical names.

phylline ethylenediamine) and theocin<sup>2</sup> (theophylline sodium acetate), using doses of equi-theophylline content. The tests were made in spastic states of the bronchi produced by different agents and in anaphylactic shock. The results obtained are summarized in this report.

**ISOLATED LUNGS Method.** The method used was essentially that described by Sollmann and von Oettingen (10) with the modifications introduced by Tainter, Pedden and James (11). In this method the perfusion fluid enters the lungs through a cannula in the trachea, and leaves the organs through minute abrasions on the lung surface. The outflow is measured in cc. per minute.

Under these conditions the bronchi at the outset are dilated. In order to test for bronchodilator action it is necessary to provoke bronchoconstriction with some drug, such as histamine or barium, which directly stimulates the bronchial muscle, or with pilocarpine, which stimulates indirectly through parasympathetic innervation. There was considerable variation in the reactions of individual lungs.

If the bronchoconstriction was too strong, the perfusion of warmed fluid was greatly reduced and the temperature of the lung decreased, making recovery and comparison more difficult. Every precaution was used to keep all conditions as constant as possible, including rate of initial outflow and speed of drug injection. In this way fairly accurate comparisons of the different bronchodilatations could be made from one set of lungs to another. The volume injected each time into the rubber tubing near the tracheal cannula varied between 0.5 and 2 cc., and the speed of injection was from 5 to 20 seconds.

The aminophyllin was used in 1 to 5% strengths (0.8 to 4% anhydrous theophylline) in 0.85% sodium chloride solution, and theocin in 1 to 4.2% strength (0.6 to 2.5% anhydrous theophylline). The dosage of both theophylline derivatives used in isolated and intact lungs was expressed in mgm. of anhydrous theophylline throughout. Forty-nine lungs were perfused in as many experiments, but only one bronchoconstrictor drug was injected in each pair of lungs. Figure 1 illustrates typical reactions of perfused bronchi of untreated and anaphylactic lungs to bronchoconstrictors and to the theophylline derivatives and epinephrine.

**Untreated lungs.** Aminophyllin and theocin, in doses of 3.2 to 32 mg., always produced an increase in the outflow. This increase varied between 5 and 120% of the outflow per minute before the injection. The greatest bronchodilatation was observed in those lungs which showed a progressive bronchoconstriction soon after perfusion was started. In 6 to 10 minutes after the injection of the theophylline derivatives the outflow was re-established at a higher and more constant level. When this level was reached and maintained, the further effect of the theophylline derivatives was less conspicuous. Sometimes the same dose of aminophyllin or theocin produced different degrees of dilatation. Usually the effect was greater at the beginning of the perfusion, the net result being that aminophyllin and theocin showed practically the same bronchodilator efficiency (fig. 1).

**Histamine bronchoconstriction.** When histamine acid phosphate (1:5000 to 1:10,000 in 0.85% sodium chloride solution) in doses of 0.02 to 0.125 mg. was injected, a rapid and intense diminution in outflow occurred, which in some lungs was nearly 100%. In these lungs the circulation of fluid was almost negligible, with no possibility of a rapid removal of the bronchoconstrictor poison spontaneously, or by the action of a bronchodilator like aminophyllin or epinephrine. The constriction lasted too long (25 to 30 minutes) and afterwards recovery

was incomplete and sensitivity to the drugs greatly reduced. There was much individual variation in these histamine reactions, but in a given lung preparation the responses to the same dose of histamine, injected at the same speed, were comparable within narrow limits.

In order to observe the bronchodilator efficiency of the theophylline derivatives, the following standard procedure was adopted: (1) injection of a broncho-

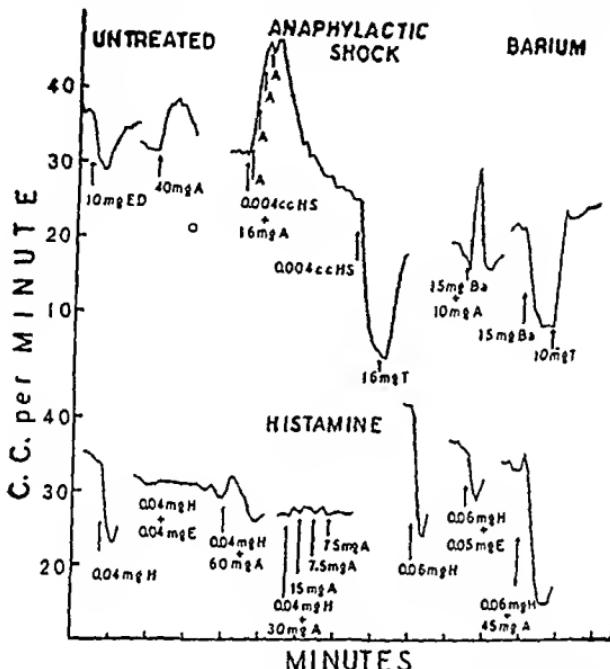


FIG. 1. TYPICAL BRONCHIAL TONUS CHANGES IN ISOLATED GUINEA PIG LUNGS

*Abscissa:* Time in periods of 10 minutes. *Ordinate:* rate of outflow in cc. per minute. The doses of drugs and the figure. A = Aminophyllin and T = Theoein, each as ~~10 mg.~~ ; H = Histamine; HS = horse serum; ED = Ethylenediamine hydrochloride. *Untreated lungs* (Exp. 8): Bronchoconstriction due to ethylenediamine and bronchodilatation due to aminophyllin. *Anaphylactic shock* (Exp. 21): Prevention of bronchospasm, and bronchodilatation, following a mixture of antigen and aminophyllin of aminophyllin; recovery time, 20 minutes; bronchoconstriction alone. Relief of the eosinophilia by aminophyllin and theoein. *Barium* (Exp. 16, ~~17~~): *Barium* relieved by aminophyllin and theoein. *Histamine* (Exp. 42-3 records at left): First, bronchoconstriction after 0.04 mg. histamine alone; second, mixtures with epinephrine or aminophyllin in repeated doses. Exp. 44 with aminophyllin in repeated doses. Exp. 44 after 0.06 mg. histamine alone; second, incomplete with 0.05 mg. epinephrine, and third, failure to prevent bronchospasm after 5 mg. aminophyllin.

constrictor dose of histamine with a reduction in outflow of 35 to 75%; (2) a period of recovery; (3) injection of the same dose of histamine plus a dose of aminophyllin or theoein. In nearly all lungs an antagonism between histamine and the theophylline derivative was demonstrated. The result was either a reduction of the histamine constriction (smaller decrease in the outflow), or abolition of the histamine effect (outflow remaining constant), or bronchodilat-

tion, that is, a predominance of the theophylline effect. Sometimes, when the two antagonistic drugs were injected, the immediate effect observed was a moderate increase in the outflow following by a slow decrease, which was, however, smaller than the effect of the same dose of histamine injected alone.

Both theophylline derivatives were equally effective in antagonizing the bronchoconstriction of histamine, taking into account the sensitivity of the method. For example, in Experiment 33, 37.5 mg. of aminophyllin (30 mg. anhydrous theophylline) and 50 mg. of theocin (30 mg. anhydrous theophylline) completely inhibited the bronchoconstriction of 0.02 mg. of histamine. The same doses of the two theophylline derivatives decreased to the same degree the bronchoconstriction of 0.04 mg. of histamine. Even high doses of the theophylline derivatives (32 mg. anhydrous theophylline) did not completely suppress the bronchoconstriction effect of histamine, when the latter drug was injected in doses that elicited at least a 50% reduction in the outflow. Accordingly, these theophylline derivatives must be considered as weak bronchodilators under the conditions.

*Pilocarpine bronchoconstriction.* Using the same procedure as with histamine, I found that doses of 0.8 mg. pilocarpine hydrochloride gave a bronchoconstriction of about 50 to 70%. The theophylline derivatives, in the same doses as used in histaminized lungs, antagonized the bronchoconstriction of pilocarpine in varying degrees. The spasm was prevented and also overcome by the theophylline derivatives. The results are not illustrated in figure 1.

*Barium bronchoconstriction.* Fifteen to 25 mg. of barium chloride injected into the perfusion fluid produced a fair degree of bronchoconstriction which was promptly antagonized by the theophylline derivatives, in the same doses as in histaminized or pilocarpinized lungs (fig. 1). The bronchospasms were both prevented and overcome by the theophylline derivatives, in about an equal degree.

*Anaphylactic lungs.* Normal guinea pigs were sensitized to normal horse serum by subcutaneous injection of 0.2 cc. for each animal, and they were killed about 8 weeks later and their lungs perfused.

Normal horse serum had little or no demonstrable effect on the bronchi of perfused lungs of normal (non-sensitized) guinea pigs, a small bronchoconstriction of about 10% reduction of the initial outflow being the greatest effect from a dose of 0.5 cc. However, in the perfused lungs of sensitized animal, the injection of antigen, even in minute doses, caused a marked bronchoconstriction which almost stopped the outflow. When this intense bronchoconstriction had been established, as after high doses of histamine, the lungs were less sensitive to the action of drugs. Recovery was not complete, but it was possible to produce a fairly strong bronchoconstriction by the injection of a fresh dose of antigen. This second, and sometimes a third, anaphylactic bronchoconstriction, with the same dose of antigen, was much weaker than that produced by the first injection.

The anaphylactic bronchoconstriction produced by doses of 0.1 to 0.0004 cc. of normal horse serum was too marked to be antagonized by the doses of the

theophylline derivatives used in histaminized and pilocarpinized lungs. These doses sometimes delayed the onset of the bronchoconstriction, or decreased it to a slight degree. However, if the dose of aminophyllin or theocin was increased and perfusion was continued for some minutes, the anaphylactic bronchoconstriction was totally suppressed and even replaced by a bronchodilatation (fig. 1).

*Ethylenediamine.* Injections of 8 to 10 mg. ethylenediamine, the component of aminophyllin, produced a slight decrease in outflow of several lungs. This reduction was about 5%. In all lungs, when this drug was injected mixed with the dose of a bronchoconstrictor drug, the effect was a slight increase of the constrictor effect, if anything. Injection of a mixture of the antigen and this amine into lungs of a sensitized guinea pig resulted in a constriction of about 100 per cent. These results, therefore, showed a lack of bronchodilator action, and any antagonism to bronchoconstriction, by ethylenediamine. Clearly the ethylenediamine has no part in the bronchial antispasmodic action of aminophyllin. It merely serves the purpose of making a more soluble theophylline compound.

*Comparative bronchodilator actions of theophylline and epinephrine.* It was difficult to make a quantitative comparison of the bronchodilator efficiency of theophylline (as aminophyllin) and epinephrine in antagonizing the bronchoconstriction of small doses of histamine. The effects of epinephrine and histamine lasted longer than those of aminophyllin. The result of injecting histamine and aminophyllin, in a ratio of 1:750 to 1:1500, was that the action of aminophyllin might prevail during the first 2 or 3 minutes. Thus, a small increase in the initial outflow would be observed, but later the histamine bronchoconstriction prevailed. Using the initial aminophyllin antagonism as a basis for comparison with the epinephrine antagonism of histamine in 11 lungs, it was found that epinephrine was about 1250 to 1870 times stronger than aminophyllin (1000 to 1500 times stronger than anhydrous theophylline). In 4 lungs which showed a moderate degree of bronchoconstriction after smaller doses of histamine, epinephrine in doses 900 to 1000 times smaller than those of aminophyllin produced the same degree of bronchodilatation. According to these criteria, epinephrine is at least 1000 times more powerful than aminophyllin in bronchospasm resulting by direct stimulation of the bronchial muscles by histamine. This agreed, in general, with the results on bronchi *in situ*.

**BRONCHI IN SITU.** *Method.* Seventeen dogs, weighing from 7 to 13 kg., were used. Eleven were injected previously with 20 mg. of morphine hydrochloride per kg., and later anesthetized with ether. The brain and the medulla were pithed, and in some animals, also the spinal cord. Five dogs were anesthetized with pentobarbital, 35 mg. per kg. intraperitoneally, and later injected with curare, 3 mg. per kg., or fluidextract of erythrina 0.1 cc. per kg. intravenously, repeating the curare or erythrina as needed. One dog was injected with morphine, 20 mg. per kg. subcutaneously, anesthetized with ether, and later eurarized with erythrina.

Blood pressure was recorded from a mercury manometer attached to the carotid artery. Injections were made in the saphenous or the jugular vein. Jackson's well known method (12) was used for recording changes in bronchial tone. Artificial respiration was by means of negative pressure. Bronchoconstriction was indicated on the kymograph record by a

decrease in the excursions of a tambour connected to the tracheal cannula. Both vagi were cut in the neck before pithing. Atropinization with complete paralysis of the vagi did not affect the actions of the bronchodilator drugs. In unpithed animals, the vagi were left intact.

The injections were washed into the vein with 5 to 10 cc. of warm solution of 6% acacia containing 5% dextrose. A slow injection of 8 to 10 cc. per kg. of the acacia solution was continued after pithing, when the blood pressure fell and the bronchoconstrictor agents were injected.

Histamine, pilocarpine, and barium chloride were used as typical bronchoconstrictors. The doses of histamine used ranged from 0.1 to 0.3 mg. per kg., with resulting prompt and marked bronchoconstriction. Sometimes the respiratory excursions were reduced more than 80%, and the blood pressure was markedly lowered at the same time. Pilocarpine was injected in doses of 0.1 to 0.5 mg. per kg. This drug produced a fairly good and constant, though slow, bronchoconstriction, a pronounced bradycardia, and moderate initial hypotension followed by a recovery of blood pressure above the original level. Barium chloride proved to be a poor bronchoconstrictor in doses below the fatal, in confirmation of previous results by Cameron and Tainter (13). Five mg. per kg. produced an increase in the blood pressure, but had no demonstrable action on the bronchial tone; 10 mg. per kg., injected slowly, caused cardiac irregularities and slight bronchoconstriction. Typical results with 2 of the bronchoconstrictors used, histamine and pilocarpine, and the bronchodilator effects of the theophylline derivatives and epinephrine are illustrated in figures 2, 3 and 4.

*Aminophyllin and theocin.* These theophylline derivatives were injected after an experimental bronchoconstriction became constant, or earlier when the bronchospasm was too intense. The doses used were from 10 to 60 mg. per kg. These doses caused a decrease in blood pressure in all dogs. In pithed animals, doses of 20 mg. as aminophyllin or theocin had little or no antagonistic effect on the bronchoconstriction caused by fairly strong doses of histamine (0.1 to 0.3 mg. per kg.). Higher doses, i.e. 40 to 60 mg. per kg., were more effective, but never re-established the initial bronchial state (fig. 2, upper record). This lack of bronchodilator efficiency might have been due, in part, to the fact that a further lowering of blood pressure was caused by the theophylline derivatives and superimposed on the lowered blood pressure caused by pithing and histamine. That is, the severe circulatory collapse presumably decreased the functional activity of bronchial muscle. Aminophyllin and theocin, in doses of 20 to 60 mg. per kg., reduced the bronchoconstriction produced by pilocarpine. The bronchodilatation that followed was markedly prolonged, but there was not a complete restoration of the original bronchial tone. The results with aminophyllin and epinephrine in a pithed animal are illustrated in figure 2 (upper record).

In unpithed animals, theocin and aminophyllin caused bronchodilatation with smaller doses (10 mg. per kg.) than in pithed animals. The theophylline dilatation, after pilocarpine, lasted for 10 to 20 minutes. When the pilocarpine constriction was pronounced, a recurrent, slowly progressive bronchoconstriction was produced. That is, the antispasmodic action of the theophylline derivatives was not permanent. Epinephrine in doses of 0.01 to 0.02 mg. per kg. gave a more powerful, but more transitory bronchodilator effect. With histamine a recurrent bronchoconstriction did not occur although the original state was not

reached. Aminophyllin and epinephrine produced an immediate and a more sustained bronchial relaxation, but a part of the final relaxation was due to natural recovery from the histaminal constriction. The doses for the immediate antispasmodic action of histamine was about the same as for pilocarpine. In unpithed animals, the blood pressure remained at a high level throughout, and therefore, the greater effectiveness of the smaller doses of the theophylline derivatives.

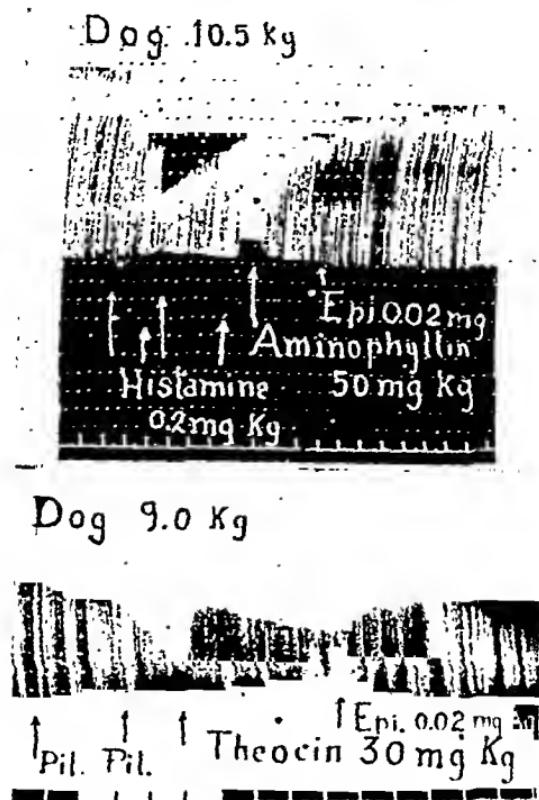


FIG. 2. COMPARATIVE BRONCHODILATOR EFFECTS OF AMINOPHYLLIN, THEOCIN AND EPINEPHRINE

*Upper record:* Dog. 10.5 kg., pithed; histamine given in 4 injections; total dose = 0.2 mg. per kg.

*Lower record:* Dog. 9.5 kg.; pentobarbital anesthesia; eurarized with erythrina. Pi. = pilocarpine hydrochloride, 1 mg. per kg. in each injection.

In all records of figures 2, 3 and 4, events read from left to right; bronchoconstriction is indicated by reduction in amplitude of respiratory excursions. Time: each stroke = 2 seconds; pil. = pilocarpine; epi. = epinephrine; amin. = aminophyllin; his. = histamine

tives was due to the maintenance of a more efficient circulation of blood through the lungs and greater functional reactivity of the bronchial muscles. There is no good reason to postulate a central action for the theophylline derivatives since identical antispasmodic actions occurred against the same types of bronchospasm in isolated perfused lungs. The various results in unpithed dogs are illustrated in figures 2 and (lower record), 3 and 4.

*Comparative efficiency of theophylline and epinephrine.* With Jackson's method it was also difficult to make quantitative determinations of bronchial tone

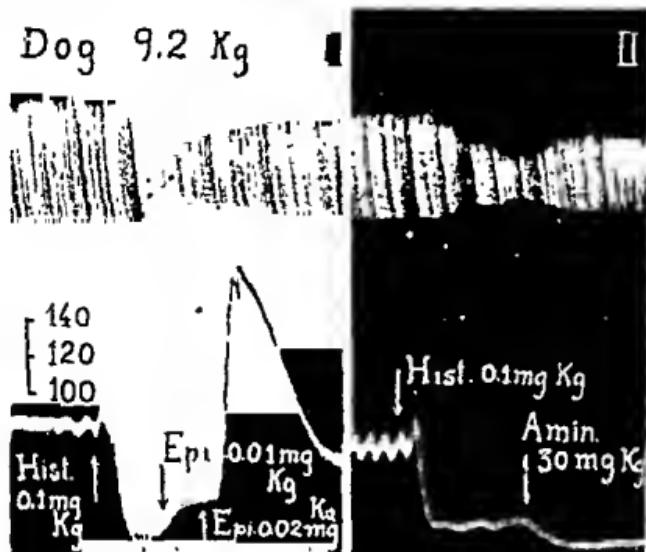


FIG. 3. COMPARATIVE BRONCHODILATOR EFFECTS OF AMINOPHYLLIN AND EPINEPHRINE ON BRONCHIAL SPASM AND BLOOD PRESSURE AFTER HISTAMINE

Dog 9.2 kg: Pentobarbital anesthesia, curarized with erythrina.  
Upper record: Bronchial tonus changes; Lower record: Changes in blood pressure (mm. Hg.).

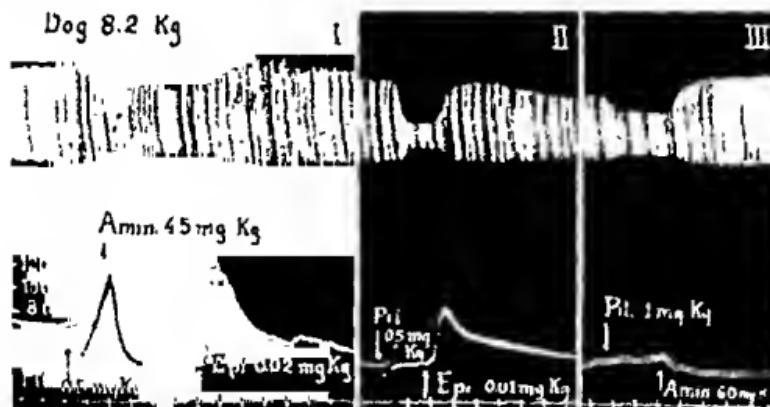


FIG. 4. COMPARATIVE EFFECTS OF AMINOPHYLLIN AND EPINEPHRINE ON BRONCHIAL SPASM AND BLOOD PRESSURE AFTER PILOCARPINE

Dog 8.2 kg: Pentobarbital anesthesia, curarized with erythrina.  
Upper record: Bronchial tonus. Lower record: Blood pressure changes. (mm. Hg.).

changes. For instance, a second dose of histamine did not produce the same degree of bronchoconstriction as did the first dose. In the same way, a second dose of aminophyllin did not produce the same degree of bronchodilatation as

did the original dose. The animals became progressively less sensitive to both bronchoconstrictors and bronchodilators. However, such comparisons as could be made justifiably indicated that both aminophyllin and theocin had about the same bronchodilator efficiency according to content of anhydrous theophylline. They could be used interchangeably for relief of bronchospasm, as in asthma. In fact, theocin would be less irritating for intravenous injection due to the fact that its sodium acetate component is less irritating than the ethylenediamine component of aminophyllin. As compared with epinephrine, both theophylline derivatives were much less efficient as to degree of bronchodilatation (figures 2, 3 and 4). That is, doses of 0.01 to 0.02 mg. per kg. of epinephrine were about as effective as doses of 40 to 60 mg. per kg. of anhydrous theophylline, whether used as aminophyllin or theocin. On a dosage basis, therefore, epinephrine was about 3000 to 4000 times stronger as a bronchial antispasmodic than was anhydrous theophylline. A similar tendency in comparative efficiency of these bronchodilators was found in perfused lungs, the epinephrine here being at least 1000 times stronger than theophylline. Clinically, the same general tendency exists in the comparative bronchodilator efficiency of these drugs. That is, the single effective dose of epinephrine in bronchial asthma is from 0.05 to 0.5 mg. for an adult and that of aminophyllin is 0.24 Gm. (0.144 Gm. anhydrous theophylline) which would make epinephrine about 288 to 2880 times stronger. When the dose of aminophyllin is doubled (0.48 Gm.), then the epinephrine would be 576 to 5760 times stronger than theophylline. It must be remembered that epinephrine is given hypodermically whereas aminophyllin is given intravenously, that is, more directly and effectively, and this, too, speaks for the greater efficiency of epinephrine.

**EFFECTS OF REPEATED DAILY INJECTION OF AMINOPHYLLIN.** Although clinical reports are silent as to possible injurious effects from repeated intravenous injections of aminophyllin in circulatory and asthmatic conditions, it is known that the theophylline derivatives are irritating. For instance, oral administration in many individuals results in gastric intolerance and in albuminuria and other manifestations of renal irritation. Intravenous injections of aminophyllin in patients cause unpleasant initial subjective symptoms, namely, prickling in the tongue, mouth, face, upper chest and arms with a marked sensation of heat in the face and neck and an irresistible quickening of respiration. In intractable asthma these symptoms are followed by a feeling of grateful relief from the dyspnea, and a diuretic effect may also ensue. The sensations of prickling and heat point to irritation of sensory nerves. Two to three such injections of aminophyllin (0.24 to 0.48 Gm.) are often given daily for a month or more, and as many as 200 such injections are known to have been given in 2 to 3 months on different occasions to one patient. Therefore it would be important to know whether such repeated injections result in permanent injuries to important viscera and especially the endothelium of the blood vessels including capillaries. If positive, a limitation should be put on the rather liberal current usage of this drug. The matter was tested by giving

repeated injections of aminophyllin in therapeutic and highest tolerated doses to rabbits and then making histological examination of different organs. The doses in this section are those of the whole molecule aminophyllin, not as anhydrous theophylline.

Doses greater than 100 mg. of aminophyllin per kg. intravenously were found to be acutely toxic or fatal. For instance, one rabbit was injected intravenously with a dose of 250 mg. per kg. and died in about 12 minutes from respiratory paralysis, after generalized convulsions. Another received 200 mg. per kg., and died in about 20 hours. Three of 6 rabbits injected with 150 mg. per kg. intravenously died within 24 hours; the other 3 survived for more than 2 weeks and were discarded. Therefore, the highest tolerated dose was assumed to be 100 mg. per kg., or about  $\frac{2}{3}$  the LD<sub>50</sub>, and this dose was actually injected each day for 30 consecutive days in 4 rabbits. Only one of these rabbits lost weight during this time. All of these rabbits were killed at the end of one month, but no macroscopic changes were found in the lungs, liver, brain, kidneys and heart.

Another group of 5 rabbits was injected intravenously daily with 7 mg. of aminophyllin per kg. for one month (30 injections in all). This dose corresponds with twice the single therapeutic dose used clinically in bronchial asthma, i.e., 0.48 Gm. total for a 70 kg. man. At the end of this time the rabbits were killed by injecting potassium chloride solution intravenously. Again no gross changes were found in the brain, heart, lungs, liver and kidneys.

Histological examinations of all organs from all rabbits were made independently by Dr. A. J. Cox, Jr. of the department of pathology, and his report is briefly summarized as follows: *Dosage of 7 mg. per kg.:* No significant meningitis; no casts in kidneys; occasional scar in livers, probably unrelated to the medication. Practically no pathological changes in any of the organs. *Dosage of 100 mg. per kg.:* Irregular, mild, chronic meningitis; small amount of granular coagulum and a few hyaline casts in the renal tubules. All other organs showed no pathological changes.

Therefore, doses of therapeutic order showed no demonstrable histological evidences of tissue irritation. However, the highest tolerated doses, which were about 30 times the single therapeutic dose, gave evidences of slight irritation in the meninges and kidneys. This, taken together with subjective evidences of irritation in patients, indicates that repeated injections of high doses of aminophyllin are somewhat injurious. Whether the injuries might be permanent or result in serious consequences is not known. While the tendency to repair is always present, human tissues are generally more sensitive than those of lower animals, and therefore, intravenous injections of aminophyllin should not be given too freely or for extended periods, or indiscriminately, despite the life-saving action of the drug.

#### CONCLUSIONS

1. The comparative bronchial antispasmodic actions of 2 theophylline derivatives, namely, aminophyllin (theophylline ethylenediamine) and theocin

(theophylline sodium acetate) and of epinephrine have been determined in isolated lungs of guinea pigs and intact lungs of dogs.

2. Aminophyllin and theocin dilated the bronchi of untreated isolated lungs, and prevented or relieved variably the bronchospasms of histamine, pilocarpine, and barium, and of anaphylactic shock. Ethylenediamine, a component of aminophyllin, had a weak bronchoconstrictor action, if anything, and did not antagonize the effects of bronchoconstrictor drugs; hence does not contribute to the antispasmodic action of aminophyllin. Epinephrine was at least 1000 times more effective than the theophylline derivatives in antagonizing bronchoconstriction in perfused lungs. According to their content of anhydrous theophylline, aminophyllin and theocin were equally effective.

3. The 2 theophylline derivatives acted identically on dogs' bronchi *in situ* and relieved promptly, though variably, the bronchospasms of histamine and pilocarpine, the results with barium being less satisfactory. Under the same conditions, epinephrine was at least 3000 times more efficient than theophylline, the bronchodilatation of the latter, however, being more sustained. This agreed with a similar tendency in perfused lungs and also in clinical effectiveness in bronchial asthma.

4. The effective bronchodilator doses of the theophylline derivatives were smaller in animals with the central nervous system intact than in the pithed, owing to more efficient blood circulation in the lungs of the former. Since the 2 drugs acted identically in isolated, and intact lungs, with innervation paralyzed the seat of the bronchial antispasmodic action of theophylline was peripheral, and due to direct depression of bronchial smooth muscle, in agreement with the known depressant action on smooth muscle in blood vessels.

5. Consecutive daily intravenous injections for 1 month in rabbits of doses of aminophyllin of therapeutic order, or 7 mg. per kg., produced no demonstrable symptoms and no pathological changes in the lungs, liver, brain and kidneys. Highest single tolerated doses or 100 mg. of aminophyllin per kg. injected similarly were fairly well tolerated symptomatically, but histologically caused evidences of a slight chronic meningitis and also a slight nephritis. These evidences of irritation were consistent with subjective manifestations of irritation in patients receiving the drug intravenously. Hence, repeated intravenous injections of large doses of aminophyllin are somewhat injurious, and this drug should not be used too freely or indiscriminately. Its irritant effects could conceivably be serious in sensitive individuals, or aggravate acute or chronic inflammatory states of the kidneys, meninges and blood vessels, but these would be minor considerations in its use in emergencies, as in intractable asthma, where aminophyllin can give dramatic and complete relief.

I wish to express my thanks to Professor P. J. Hanzlik for his suggestions, guidance and criticisms.

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# THE DEACETYLATION OF HEROIN AND RELATED COMPOUNDS BY MAMMALIAN TISSUES

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It was recently shown (1) that rabbit blood serum contains an enzyme (or enzymes) capable of catalyzing the complete deacetylation of diacetylmorphine (heroin) and monoacetylmorphine and the partial deacetylation of diacetyldihydromorphine (dihydroheroin). This report deals with the distribution of this enzymic activity in the tissues of the rabbit, rat, and human.

**METHOD.** The rate of deacetylation of the morphine derivatives was measured with Barcroft differential manometers. The method depends on the reaction of the liberated acetic acid with the bicarbonate of the Ringer's solution (2) and consequent liberation of carbon dioxide, which was measured manometrically. Diacetylmorphine, diaeetyl dihydromorphine, monoacetylmorphine, and monoacetyl dihydromorphine were used as substrates. The substrate concentration was always 5.0 mgm. in 3.0 cc. The hydrochloride salts of the morphine derivatives, dissolved in Ringer's solution, were placed in the main compartment of the reaction flask and the tissue suspension in a side arm. After flushing the flasks with 95% nitrogen and 5% carbon dioxide for ten minutes, the manometers were closed, equilibrium attained, and the tissue added to the main compartment to start the reaction. The total fluid volume was always 3.0 cc. Determinations were made at 37.5°C.

The organs to be examined were removed immediately after killing by decapitation (rats) or a blow on the head (rabbits) and kept in closed glass containers in the ice cube compartment of a refrigerator. The enzymic activity of the organs showed no appreciable change over a period of eight weeks or more when kept frozen in this manner. Two samples of human liver were obtained through the cooperation of Dr. F. Eberson, Gallinger Hospital, Washington, D. C. They were obtained and frozen within four hours post mortem. One patient died of cerebral thrombosis and the other of lobar pneumonia. Both livers were free of any special pathological processes.

In each experiment slices of the frozen tissue were rapidly weighed on a torsion balance and well ground with clean sand with additions of small quantities of bicarbonate Ringer's solution. The paste was then diluted to the desired volume. This suspension was thoroughly mixed and pipetted into the side arm of the reaction flask in the desired quantity. Only the cortex of brain or kidney was used.

The rabbits used were one- to two-year-old albinos and were fed Purina rabbit chow, cabbage, and unlimited water. Albino rats (Wistar) were used at ages varying from four to five months. The rats were fed Purina dog chow and allowed unlimited water.

The acetylated derivatives of morphine were furnished by Doctor L. F. Small of the National Institute of Health and their properties and methods of preparation were described in a previous report (1).

**RESULTS.** Figure 1 shows the observed rate of liberation of carbon dioxide when rabbit tissues, in the indicated quantities, were added to 5.0 mgm. of heroin. All of the tissues examined were able to deacetylate heroin, although at different rates for equivalent amounts of tissue. The yield of carbon dioxide in the presence of relatively large quantities of tissue (except muscle) was always

greater than the theoretical amount (1) for removal of one acetyl radical from the heroin. This fact, together with the general shape of the curves, indicates that both acetyls are split off, each at a different rate. That the 3-carbon acetyl radical is more readily split off is indicated by the rate of hydrolysis of monoacetyl morphine (fig. 2).

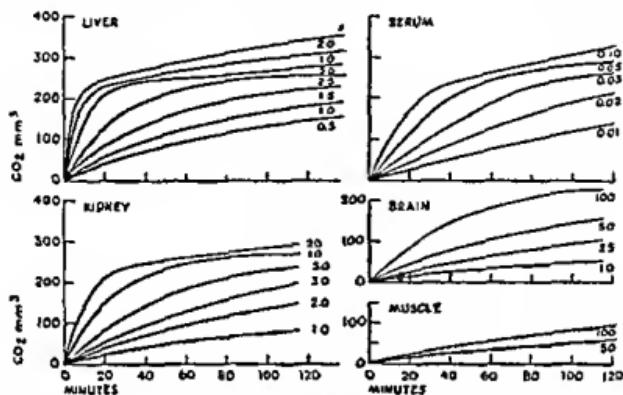


FIG. 1. THE RATE OF DEACETYLATION OF HEROIN BY DIFFERENT QUANTITIES OF RABBIT TISSUES

In this and succeeding figures the numbers on each curve represent the wet weight (mgm.) of tissue or cc. of serum used.

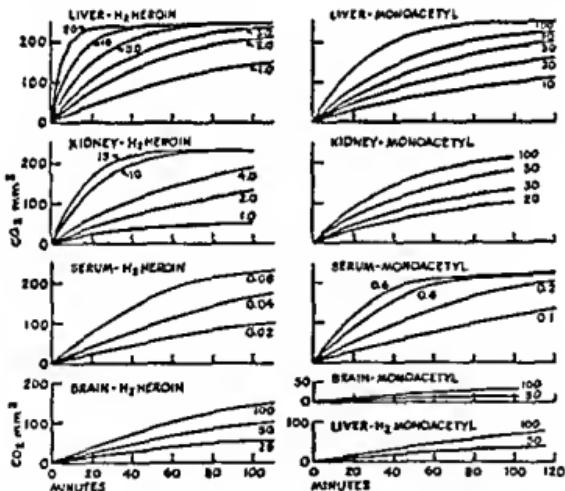


FIG. 2. THE RATE OF DEACETYLATION OF DIHYDROHEROIN, MONOACETYL MORPHINE AND MONOACETYL DIHYDROMORPHINE BY RABBIT TISSUES

The four graphs on the right of figure 2 show that the 6-carbon acetyl radical is removed from monoacetylmorphine by liver, kidney, serum, and brain. Much larger quantities of tissue were required for rates of CO<sub>2</sub> evolution comparable to those of figure 1, showing that heroin is deacetylated in two stages, at the 3-carbon first and then more slowly at the 6-carbon.

The four graphs on the left of figure 2 show the observed rate and extent of liberation of carbon dioxide when rabbit tissues were added to 5.0 mgm. of dihydroheroin. The order of ability to deacetylate at the 3-carbon is the same as found for heroin, but the rate of deacetylation is slower with equal quantities of tissue. Furthermore, the yield of carbon dioxide and general shape of the curves indicate that no measurable hydrolysis takes place at the 6-carbon with the quantities of tissue used. However, when larger quantities of liver (50 and 100

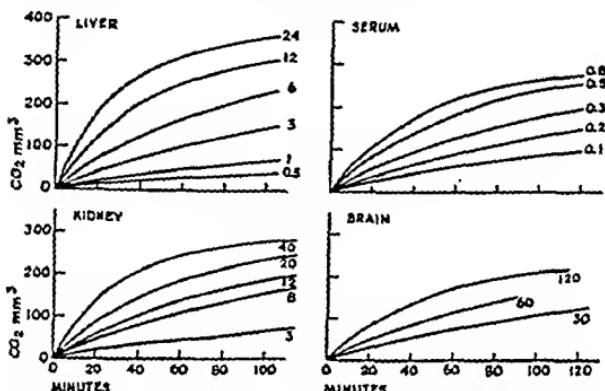


FIG. 3. THE RATE OF DEACETYLATION OF HEROIN BY RAT TISSUES

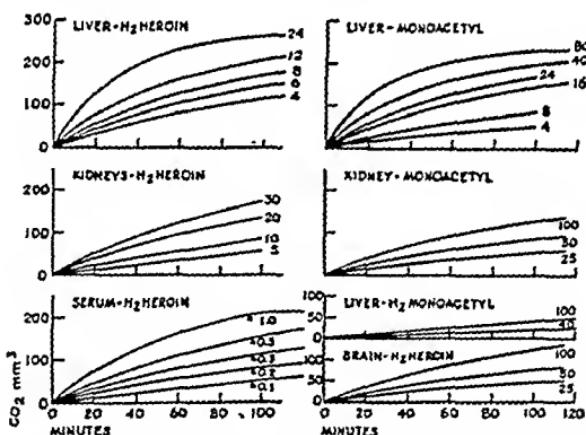


FIG. 4. THE RATE OF DEACETYLATION OF DIHYDROHEROIN, MONOACETYLMORPHINE, AND MONOACETYLDIHYDROMORPHINE BY RAT TISSUES

mgm.) were used, some deacetylation occurred at the 6-carbon, as shown in the lower right hand graph of figure 2. Similar quantities of the other tissues did not liberate carbon dioxide when added to monoacetyldihydromorphine.

The results of a series of determinations of the enzymic activity of tissues taken from a rat are given in figures 3 and 4. Figure 3 shows the rate of deacetylation of heroin when liver, kidney, serum, or brain was added to 5.0 mgm. of dihydroheroin. While all four tissues readily remove the 3-carbon acetyl radical, they do so at a slower rate per unit of tissue than do the tissues of the rabbit (figs. 1, 2). The

yield of carbon dioxide in the presence of the larger quantities of liver or kidney indicates a partial deacetylation at the 6-carbon, in other words, that these two tissues are able to remove the 6-carbon acetyl radical from monoacetylmorphine. This was proved by the liberation of carbon dioxide on addition of liver or kidney to 5.0 mgm. of monoacetylmorphine (fig. 4, upper right).

Rat liver, kidney, serum, and brain are able to split off the 3-carbon acetyl from dihydroheroin (fig. 4), but the rate of hydrolysis was slower for equal quantities of tissue, than when heroin was used as the substrate. Of the rat tissues investigated only the liver was able to measurably remove the acetyl radical from monoacetyldihydromorphine.

Human liver is able to deacetylate all four acetylated morphine derivatives (fig. 5). The observed rate of hydrolysis at the 3-carbon per unit of tissue was slower in the presence of human liver than rabbit liver and approximately equal to that of rat liver. However, the hydrolysis at the 6-carbon was somewhat

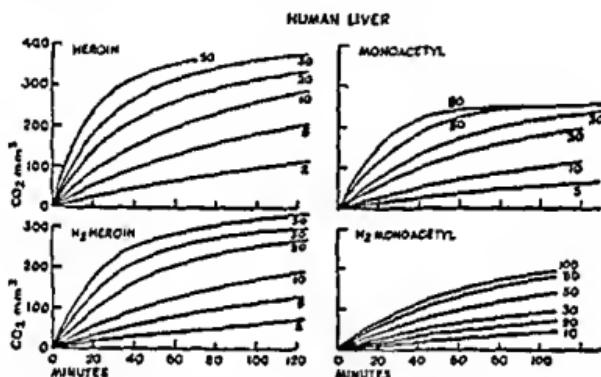


FIG. 5 THE RATE OF DEACETYLATION OF HEROIN, DIHYDROHEROIN, MONOACETYLMORPHINE AND MONOACETYLDIHYDROMORPHINE BY HUMAN LIVER

faster with human liver than rabbit or rat liver as can be seen by a comparison of the rates of deacetylation of monoacetylmorphine or monoacetyldihydromorphine by these tissues (figs. 2, 4, and 5).

The initial rate of hydrolysis of monoacetylmorphine or monoacetyldihydromorphine, representing the removal of the 6-carbon acetyl, followed fairly well the rate to be expected for a monomolecular reaction. This is shown by the two graphs at the right of figure 6. Here the logarithm of  $(a - x)$  from the equation:—

$$K = \frac{2.3}{t} \log \frac{a}{(a - x)}$$

is plotted against minutes. The initial substrate concentration ( $a$ ) is expressed in equivalents of carbon dioxide ( $\text{mm}^3$ ),  $x$  represents carbon dioxide evolved at a given time ( $t = \text{minutes}$ ) and  $K$  is the velocity constant, determined from the slope of the line.

In the same way, values for the velocity constant for hydrolysis at the 3-carbon of heroin and dihydroheroin can be derived if the quantity of tissue used is

so small that no appreciable hydrolysis takes place at the 6-carbon or if the initial slope of the line is taken when larger quantities of tissue are used (fig. 6).

The value chosen for  $(a)$  was  $240 \text{ mm}^3$  in each case. While this is less than the theoretical amount (approx.  $275 \text{ mm}^3$ ) for complete deacetylation at one carbon, it does represent the average value found experimentally and also is the best value found by extrapolation to zero time when  $\log(a-x)$  is plotted against minutes (fig. 6).

The values found for  $K$  using different quantities of rabbit, rat, and human tissues are given in table 1. These values were derived from the data plotted in figures 1 to 5. Inspection of the tables shows that the velocity constants are very

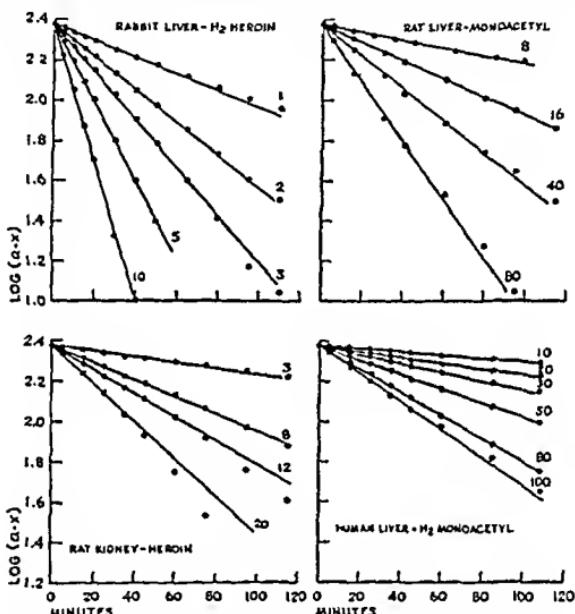


FIG. 6. THE LOGARITHM OF THE SUBSTRATE CONCENTRATION ( $\text{mm}^3\text{CO}_2$ ) AT INTERVALS AFTER MIXING THE TISSUE AND SUBSTRATE INDICATED

nearly proportional to the amount of tissue used. Therefore, a comparison of the velocity constant should give the relative enzymic activities of the tissues.

In order to determine the amount of tissue required to give equal velocity constants, in other words containing equal enzymic activity, the values of  $K$  given in the tables were plotted against milligrams of tissue and a straight line drawn through the points. The amount of each tissue required to give a velocity constant of 0.01 was obtained, extrapolating where necessary. These quantities are given in table 2 and represent the amount of tissue required to hydrolyze 1% of the substrate per minute. The values given for heroin and dihydroheroin are for hydrolysis at the 3-carbon, while those for monoacetylmorphine and monoacetyldihydromorphine are for 6-carbon hydrolysis.

From table 2 a comparison of the enzymic activity of the tissues can be obtained by direct comparison of the milligrams of tissue given. Thus the enzymic

TABLE 1.

*The variation of the velocity constant (K) with tissue concentration*

In this and following tables, Heroin and H<sub>2</sub> heroin represent hydrolysis at carbon-3 and monoacetyl and H<sub>2</sub> monoacetyl at carbon-6. Substrate concentration = 5.0 mgm. in 3.0 cc.

	LIVER		KIDNEY		BRAIN		SERUM				
	Mg.	K × 10 <sup>3</sup>	Mg.	K × 10 <sup>3</sup>	Mg.	K × 10 <sup>3</sup>	Mg.	K × 10 <sup>3</sup>			
Rabbit											
Heroin	0.5	0.8	1.0	0.36	10	0.22	10	0.60			
	1.0	1.6	2.0	0.78	25	0.49	20	1.14			
	1.5	2.2	3.0	1.2	50	0.93					
	2.5	3.7	5.0	2.1	100	2.2					
H <sub>2</sub> heroin	1.0	0.94	2.0	0.76	25	0.23	20	0.5			
	2.0	1.9	4.0	1.43	50	0.46	40	1.0			
	5.0	4.5	10.0	4.1	100	0.86	80	2.1			
Monoacetyl morphine	20	0.83	20	0.59	50	0.05	100	0.59			
	30	1.4	30	0.86	100	0.10	200	1.3			
	100	3.8	50	1.4			400	2.5			
H <sub>2</sub> monoacetyl morphine	50	0.16	N	N	N	N	N	N			
	100	0.34									
Rat											
Heroin	1.0	0.32	3.0	0.32	30	0.54	100	0.41			
	3.0	0.87	8.0	0.97	60	1.0	200	0.70			
	6.0	1.7	12.0	1.3	120	2.0	300	1.1			
H <sub>2</sub> heroin	4.0	0.65	10	0.45	25	0.20	200	0.41			
	12	1.9	20	0.84	50	0.37	500	0.97			
	24	3.8	30	1.2	100	0.72	1000	1.0			
Monoacetyl morphine	8.0	0.49	25	0.26	100	0.078					
	16	1.1	50	0.44							
	40	1.9	100	0.80							
H <sub>2</sub> monoacetyl morphine	40	0.09	N	N	N	N	N	N			
	100	0.20									
Human liver											
	HEROIN		H <sub>2</sub> HEROIN		MONOACETYL MORPHINE		H <sub>2</sub> MONOACETYL MORPHINE				
	Mg.	K × 10 <sup>3</sup>	Mg.	K × 10 <sup>3</sup>	Mg.	K × 10 <sup>3</sup>	Mg.	K × 10 <sup>3</sup>			
	2	0.52	5	.65	10	.59	20	.36			
	5	1.3	10	1.1	20	1.3	30	.52			
	10	2.5	20	2.7	30	2.0	50	.66			
					50	3.2	50	1.3			

activity of rabbit liver, as regards hydrolysis at the 3-carbon of heroin, was 6 times as great as rat liver and seven times as great as human liver. A similar comparison with dihydroheroin as substrate showed rabbit liver six to seven times as active as rat or human liver. However, when a comparison is made of the activity of the same livers using monoacetylmorphine as substrate, the enzymic activity of the human liver is equal to or greater than that of the rat or rabbit. When monoacetyldihydromorphine was used as substrate, the human liver was approximately eight times as active as the rabbit or rat liver. The observation that the rat and human liver samples were approximately equal in ability to deacetylate monoacetylmorphine, but that the human liver was much the more

TABLE 2

*Milligrams (wet weight) of tissue required to give a monomolecular velocity constant of 0.01*

		LIVER	KIDNEY	BRAIN	SERUM*
Heroin.....	Rabbit†	0.62	2.6	48	17
	Rat‡	3.5	8.5	58	270
	Human§	4.0	—	—	1,100
H <sub>2</sub> heroin.....	Rabbit	1.1	3.0	110	38
	Rat	6.2	12.0	140	515
	Human	7.5	—	—	—
Monoacetylmorphine.....	Rabbit	23	35	1,000	160
	Rat	19	115	1,300	N
	Human	16	—	—	—
H <sub>2</sub> monoacetylmorphine.....	Rabbit	450	N	N	N
	Rat	500	N	N	N
	Human	61	—	—	—

All subjects from which these tissues were obtained were males.

\* 1.0 cc. of serum = 1 gram. N = No hydrolysis with 0.5 gram of tissue.

† Rabbit #294.

— = No examination of tissue made.

‡ Rat #1.

§ Subject A.

active when splitting monoacetyldihydromorphine was confirmed by repeated tests.

A comparison of the quantities of different tissues required for equal values of K (table 2) shows that liver has the greatest capacity for deacetylation either at the 3- or 6-carbon position. Kidney is next in order of activity followed, in the rat, by brain and then serum. In the rabbit the order of activity of brain and serum is dependent on the activity of the individual rabbit's serum. That this varies widely has been shown previously (1) and is confirmed by the figures in table 3. The few determinations that have been made on muscle all showed a lower enzymic activity than the tissues listed in the tables.

The ratio of activities of one organ in two species of animals is no indication of

what the ratio will be in another organ. For example, rabbit liver is roughly six times as active as rat liver, as regards deacetylation at the 3-carbon position, but brain cortex from the two species is approximately equal in activity. The serum of the rabbit may be equal to that of the rat or sixteen times as active, due to the wide variation in the activity of rabbit serum.

TABLE 3

*The variation in amount of tissue required for a velocity constant of 0.01*

ANIMAL NO.	SUBSTRATE	LIVER	SERUM	ANIMAL NO.	SUBSTRATE	LIVER	KIDNEY	BRAIN	SERUM
Rat									
2	Heroin	2.8	150	7	Heroin	3.8	8.8	65	233
3	Heroin	2.8	140	8	Heroin	4.5	8.9		250
4	Heroin	2.8	140	9	Heroin	3.3	8.6	60	185
5	Heroin	2.5	135	10	Heroin	3.9	12		264
6	Heroin	2.3	156	11	Heroin	3.9	8.7		
6	H <sub>2</sub> heroin	3.9	330	11	H <sub>2</sub> heroin	6.7	20		
4	Monoacetyl morphine	12	N	10	Monoacetyl morphine	13	102		N
Rabbit									
277	Heroin		31	275	Heroin	0.64	1.8		208
276	Heroin		209	273	Heroin	0.63			40
277	H <sub>2</sub> heroin		73	275	H <sub>2</sub> heroin	1.1	2.3		278
276	H <sub>2</sub> heroin		290	273	H <sub>2</sub> heroin	1.3			
277	Monoacetyl morphine		260	275	Monoacetyl morphine	40			N
273	H <sub>2</sub> monoacetyl morphine	300		273	Monoacetyl morphine	24			
Human									
B	Heroin	4.0		B	Monoacetyl morphine	10			
B	H <sub>2</sub> heroin	8.0		B	H <sub>2</sub> monoacetyl morphine	42			

Animals 2, 3, 4, 5, and 6 were females; all the rest were males.

The values in table 3 are comparable to those in table 2 and show the variation in quantity of tissue required to give a velocity constant of 0.01 when the tissues are taken from different animals. The concentration of enzyme responsible for deacetylation at the 3-carbon position is definitely less in the liver and serum of the male rat than in the female rat. Both sets of animals were approximately

four months old, but the males were much larger than the females so that the total concentration of the enzyme might very well have been the same in the two sexes. Outside of this sex difference the tissues of the rat were fairly constant as regards concentration of the enzyme. In fact, rabbit serum was the only source of enzyme that gave extremely wide variations in enzymic activity.

**DISCUSSION.** From the data presented it is quite apparent that the rat, rabbit, and human are able to convert heroin to morphine at a rapid rate by means of the liver alone. For example, 4.0 mgm. of human liver remove 1% of the 3-carbon acetyl from 5 mgm. of heroin in one minute (table 2). Assuming a direct proportionality between the quantity of liver and the velocity of hydrolysis over a wide range, it would require only 360 mgm. of liver to deacetylate 90% of the heroin at the 3-carbon in one minute. The same rate of removal of the 6-carbon acetyl would take approximately four times as much tissue, but even at that the limiting factor for conversion of injected heroin would probably be the time taken for the drug to arrive at the liver by means of the circulation. The rate of deacetylation of heroin by the liver of a rat or rabbit would be of the same general order of that for human liver.

In view of these quite probable rapid rates of conversion of heroin to morphine by the liver, it is difficult to avoid the conclusion that injected heroin exerts most of its pharmacological action as morphine. This is especially true since the kidney, brain, and serum are also contributing to the hydrolysis of the heroin.

The same possibility applies to deacetylation of dihydroheroin at the 3-carbon. However, the rate of deacetylation of this compound at the 6-carbon, by the rat or rabbit, would be considerably slower. This might account for the less intense pharmacological action (3, 4) of dihydroheroin and monoacetyldihydromorphine.

Rizzoti's observation (5) that beating frog hearts convert heroin to monoacetylmorphine and, in part, to morphine was brought to my attention after completion of the work on deacetylation of heroin by rabbit serum (1). Rizzoti found no hydrolysis of heroin in contact with non-functioning hearts or extracts of heart tissue and concluded that the hydrolysis was intimately connected with cardiac contraction.

A number of determinations were made of the cholinesterase activity of the tissues examined for hydrolysis of heroin. The order of activity for rat tissues with acetylcholine as substrate was: brain > liver > serum > kidney. Brain had as much as twenty times more cholinesterase activity than kidney in some of the animals. The order of activity for deacetylation of the morphine derivatives in the same animals was: liver > kidney > brain > serum. This is further evidence (1) that cholinesterase is not involved in the deacetylation of heroin, either at the 3-carbon or 6-carbon position.

Bernheim and Bernheim (7) found that blood serum from only a limited number of rabbits was able to hydrolyze atropine. The same is true for the hydrolysis of monoacetylmorphine. Examination of blood serum from a number of rabbits has shown an exact parallelism in ability to hydrolyze atropine and monoacetylmorphine. This indicates that the same esterase is involved for hydrolysis of atropine and for hydrolysis at the 6-carbon position of the acetyl-

lated morphine derivatives. However, Glick and Glauhach (8) found in the rabbit that serum is the richest source of "atropinesterase", and that when the atropine-splitting enzyme was absent from the serum, it was also absent from the tissues. This is not true for the hydrolysis of monoacetylmorphine.

#### SUMMARY

Mammalian tissues (human, rabbit, and rat) contain an enzyme or enzymes capable of deacetylating heroin and dihydroheroin at both the 3-carbon and 6-carbon positions. Of the tissues examined, liver has the highest concentration of this enzymic activity followed, in general, by kidney, brain, blood serum, and muscle. Dihydroheroin is deacetylated at a slower rate per unit of tissue than heroin and the 6-carbon acetyl is removed from both at a much slower rate than the 3-carbon acetyl. Rabbit liver has a greater capacity for deacetylation at the 3-carbon position, than rat or human liver while human liver deacetylates at the 6-carbon position more rapidly than rabbit or rat liver. There is some evidence that the enzyme that catalyzes the hydrolysis of atropine also catalyzes the hydrolysis of monoacetylmorphine or the hydrolysis of heroin at the 6-carbon position.

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# THE DEVELOPMENT OF TOLERANCE TO DEMEROL<sup>1</sup>

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It is well known that when repeated doses of any of the opiates are given for the relief of pain, tolerance to the analgetic action is developed. This is evidenced by the fact that equal doses produce diminishing effects, hence larger doses are required to obtain the original therapeutic action. Some studies on Demerol (1, 2) indicate that certain aspects of its action closely resemble those of morphine. For this reason it seemed desirable to determine to what degree tolerance might be developed to repeated doses.

**METHODS.** Four prisoner patients who had been addicted to opiates, but who had received no drugs for at least nine months, volunteered for the study. All of these men were serving sentences long enough to insure complete recovery between the end of the study and their release.

The method of Hardy and Wolff (3) for determining pain thresholds was used. The only change from their original method was the use of a wattmeter in place of a radiometer for measuring the stimulus intensity (4). Before the start of the present study all of the men had been familiarized with the experimental procedure and had proved to be good subjects.

Demerol was administered subcutaneously in initial doses of 100 mgm. and the effect on the pain threshold of this dose was determined. After the first dose the men chose the size and frequency of their doses to meet their particular desires, with limits of 300 mgm. per dose and a minimum interval of 1½ hours between doses. All of them took the drug at fairly regular intervals in gradually increasing doses. A pain threshold determination was made each week on three of the patients, and at less regular intervals on the fourth. Each run was started at least 1½ hours after the preceding dose of Demerol. When the "preinjection" threshold had been obtained the dose then being chosen by the patient was administered and determinations made every fifteen minutes until the threshold returned to the pre-injection level.

The drug was temporarily withdrawn after eight weeks of administration and the weekly pain threshold determinations were discontinued. The abstinence syndrome following complete withdrawal has been described by Himmelsbach (5). Measurements were made at 15-day intervals following withdrawal, using 100 mgm. as a test dose.

**RESULTS.** The magnitude of the pain-threshold-raising effect of the initial dose of 100 mgm. was different in each of the four cases and no attempt has been made to average the results. In each case, however, there was a marked reduction in the effect as the study progressed. Figure 1, showing some of the data obtained from patient 1, is typical. It is evident that tolerance to the drug developed rapidly and that at the end of eight weeks there was a reduced response from a dose considerably greater than that originally used.

It will be noted (figure 1) that as tolerance developed the time at which the

<sup>1</sup> 1-methyl-4-phenyl-piperidine-4-carboxylic acid ethyl ester, known in Europe as Dolantin. This compound was supplied through the courtesy of Doctor O. W. Barlow of the Winthrop Chemical Company.

<sup>2</sup> Biophysicist, United States Public Health Service.

threshold raising effect reached a maximum decreased progressively. Since it is probable that this time depends to some extent upon the relation between the rate of absorption and the rate of removal of the drug, it appears that tolerance is accompanied by, or is the result of, changes in the rate of drug utilization.

PAIN THRESHOLD RAISING EFFECT OF DEMEROL

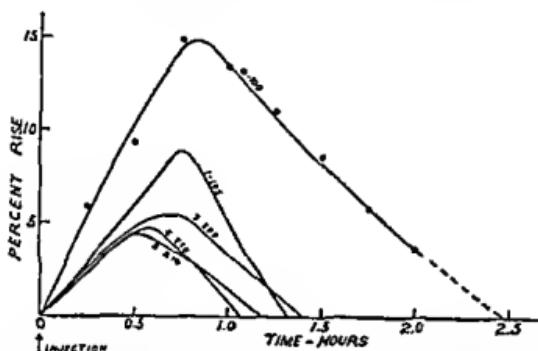


FIG. 1. DATA OBTAINED FROM PATIENT I SHOWING THE PERCENTAGE RISE IN PAIN THRESHOLD AFTER THE ADMINISTRATION OF DEMEROL

The figures near each curve give first the number of weeks of regular administration of Demerol and second, the dose in mgm. For example, the 3-200 curve was obtained after three weeks of administration using a dose of 200 mgm.

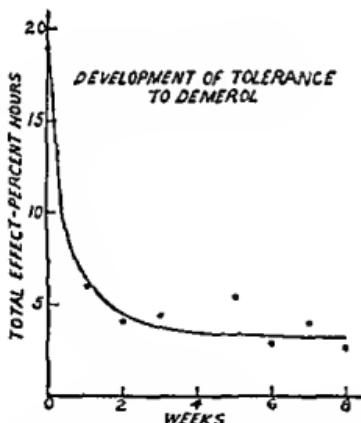


FIG. 2. THE REDUCTION IN THE TOTAL EFFECT (AREA UNDER THE PERCENTAGE RISE-HOURS CURVES) IN PATIENT I

Each value of total effect is plotted against the number of weeks of regular administration for that particular determination.

If the area under each of the percentage rise-hours curves is measured the total threshold raising effect of the particular dose used is obtained. This total effect will be expressed in per cent hours. In figure 2 the values of the total effect thus obtained are plotted against the number of weeks Demerol had been administered. This total effect curve indicates that tolerance developed quite rapidly and had nearly reached a maximum at the end of eight weeks.

It is interesting to note that the preinjection threshold readings, taken just before the administration of the test dose and at least  $1\frac{1}{2}$  hours after the preceding dose, showed no significant changes (table 1).

The most striking result was the failure of a single test dose (100 mgm.) to produce a threshold-raising effect for at least 30 days after withdrawal. At this time there was no appreciable effect in any of the cases studied. After 45 days of abstinence one case continued to show no effect, two showed a small, irregular effect, and one showed a response almost equal to that obtained from the first dose.

TABLE 1  
*Pre-injection threshold readings*

WEEKS OF ADMINISTRATION	PRE-INJECTION THRESHOLD			
	Patient I	Patient II	Patient III	Patient IV
0	289	267	277	266
1	277	258	269	
2	271	253	273	279
3	267	267	267	
5	263	249	272	
6	286	265	268	
7	256	262	262	
8	263	264	256	256
DAYS AFTER WITHDRAWAL				
15	263	270	285	
30	301	273	267	
45	301	270	270	

DISCUSSION. The development of tolerance to repeated doses of Demerol was not unexpected, for in many ways the action of this compound closely resembles that of morphine. It was surprising, however, to find that this tolerance was maintained for so long a time after withdrawal. Clinical recovery appeared to be almost complete after 30 days of abstinence, yet at this time no case showed a significant increase in threshold following a single dose of Demerol. It has been found that with morphine the development of tolerance may be almost completely irreversible, for when a single dose of morphine is given to an individual who has previously been addicted, the threshold raising effect is in general absent, or greatly reduced below that obtained in a normal with the same dose. The tolerance developed to Demerol is maintained beyond the period of administration but appears to be less permanent than that developed to morphine.

#### SUMMARY AND CONCLUSIONS

Using patients previously addicted to morphine, it has been shown that tolerance develops to the pain threshold raising effect of Demerol. The development

of tolerance appears to be nearly maximal at the end of 8 weeks. There was no significant change in the *pre-injection thresholds*. The tolerance is maintained for at least 30 days after the drug has been discontinued.

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# A COMPARISON OF THE OFFICIAL AND THE CHICKEN METHODS FOR THE OXYTOCIC BIOASSAY OF POSTERIOR PITUITARY PREPARATIONS<sup>1,2</sup>

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The purpose of this investigation was to compare the chicken blood pressure and the official uterine methods for the oxytocic bioassay of posterior pituitary preparations.

The present official method (1) is essentially that described by Dale and Laidlaw (2) in 1912. It has not been uniformly successful in the hands of all bioassayists and has been criticized by many workers from Hamilton and Rowe (3) in 1916 to Morrell, Allmark, and Bachinski (4) in 1940.

The chicken blood pressure depressor method was introduced in 1939 by Coon (5), who claimed many advantages for it. This method is based on the fact, first described by Paton and Watson (6) in 1912, that the intravenous injection of posterior pituitary solution elicits a precipitous and fleeting fall of blood pressure in the bird. This response was shown by the same workers to be accompanied by a splanchnic vasodilatation. Hogben and Schlapp (7) showed that this effect was not due to histamine or histamine-like substances, and Hogben (8) proved the ability to elicit the avian depressor response to be a specific property of posterior pituitary extracts. Following the separation of the pressor and oxytocic principles by Kamm and his associates (9), Gaddum (10) and subsequently Morash and Gibbs (11), Holtz (12), and Dictel (13), found the response to be produced by smaller doses of pitocin than of pitressin. This observation was used by Geiling (14) and by Sealock and duVigneaud (15) for estimating oxytocic activity, but Coon was the first to determine the conditions for its use as the basis for a reliable method of bioassay.

The question naturally arises as to the agreement of the two methods. Coon found that they agree for preparations such as solution of posterior pituitary and pitocin in which the pressor-oxytocic ratio is one or less. He also found, however, that the results of the two methods disagree for preparations which have a pressor-oxytocic ratio greater than 2.5:1. In such cases the discrepancy may reach 400% increasing with the pressor-oxytocic ratio. As Coon pointed out, there was no evidence to indicate which of the methods was at fault.

In view of the advantages claimed for the chicken method, it appeared worth while to investigate the technic thoroughly in order to discover the factors responsible for the discrepancy in results noted above.

<sup>1</sup> Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Chicago.

<sup>2</sup> A report covering part of this work was presented before the American Society for Pharmacology and Experimental Therapeutics on April 19, 1941.

EXPERIMENTAL. Pituitary preparations having pressor-oxytocic ratios ranging from 1:15 to 30:1 were assayed by both the official and the chicken methods for oxytocic activity. The two methods agreed for preparations having pressor-oxytocic ratios ranging from 1:15 to 3.5:1. With preparations having ratios ranging from 3.5:1 to 30:1 the chicken method gave higher results; the discrepancy varied with the pressor-oxytocic ratio (fig. 1) and reached 350% in the case of pitressin, which had a ratio of 30:1. These figures differ slightly from those of Coon but sustain his conclusion that "the pressor component must exceed the oxytocic component by more than 250% before it will introduce significant discrepancy in assay results by the two methods." The results admit of two possible explanations. Either the presence of relatively large amounts of pressor principle augments the depressor response, causing the chicken method to give high results; or such a preponderance of pressor principle antagonizes the

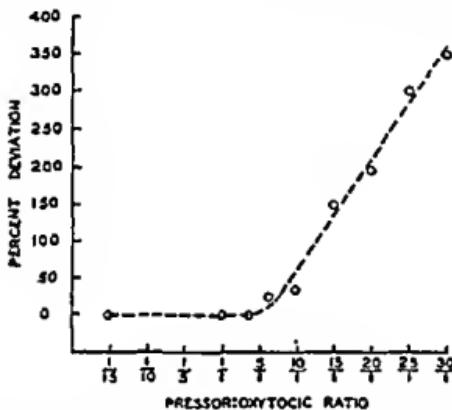


FIG. 1. CURVE EXPRESSING THE RELATION BETWEEN THE PRESSOR-OXYTOCIC RATIO OF THE PREPARATIONS AND THE PERCENTAGE DEVIATION OF THE CHICKEN ASSAY VALUES FROM THOSE OBTAINED BY MEANS OF THE OFFICIAL METHOD

action of the oxytocic principle on the isolated guinea pig uterus, causing the uterine method to give low assay values.

In order to discover which method was at fault the following experiment was carried out. Samples of pitressin and pitocin\* were assayed by both methods for oxytocic activity, and for pressor activity on the anesthetized cat. From these two preparations mixtures were prepared having known oxytocic and pressor potencies and, therefore, known pressor-oxytocic ratios, the latter ranging from 1:15 for pitocin to 30:1 for pitressin. These mixtures were then assayed for oxytocic activity by both methods. The results are shown in fig. 2. The increase of the pressor-oxytocic ratio of pitocin due to admixture with pitressin in varying proportions did not alter the oxytocic values of the mixtures as determined by the official uterine method. Therefore it is apparent that the presence

<sup>3</sup> The author wishes to express his appreciation to Dr. Oliver Kamm of Parke, Davis and Company for supplying these materials.

of the pressor principle in preponderant amounts does not inhibit the action of the oxytocic principle on the isolated uterus. It appears, then, that the presence of relatively large quantities of pressor principle in preparations having high pressor-oxytocic ratios augments the avian depressor response to the oxytocic component of such preparations.

In 1939, Fraser (16) reported that postlobin-V (pressor) appeared four times as active when compared with postlobin-O (oxytocic) by the uterine method, using the Van Dyke-Hastings modification of Tyrode's solution, as it did when compared with the same standard by the same method using Locke's solution. He found that the high magnesium concentration of the Van Dyke-Hastings solution, 2.5 mgm. %, was the factor responsible for the discrepancy, and that when the magnesium was replaced with its osmotic equivalent of sodium chloride

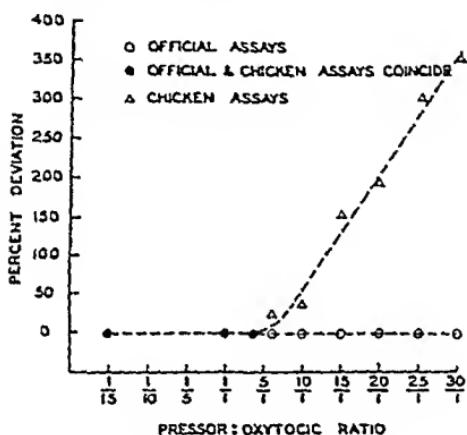


FIG. 2. CURVE ILLUSTRATING THE PERCENTAGE DEVIATION OF OFFICIAL AND CHICKEN ASSAY VALUES OF SOLUTIONS WITH VARYING PRESSOR-OXYTOCIC RATIOS FROM THE VALUES CALCULATED FROM THE OFFICIAL POTENCIES OF THE PITOCIN AND PITRESSIN FROM WHICH THEY WERE PREPARED

the assay values corresponded with those obtained when Locke's solution was used.

The official Locke-Ringer solution contains only a trace of magnesium, and it appeared possible, in the light of Fraser's findings, that the magnesium concentration of chicken blood serum might be responsible for the observed high oxytocic values of pressor preparations when assayed by the chicken method. According to Eveleth (17), the average serum magnesium level of chickens is 2.8 mgm. %. In order to test this hypothesis, solutions with varying pressor-oxytocic ratios which had already been assayed by the official and chicken methods were reassayed by the uterine method, using the official Locke-Ringer solution with the magnesium concentration adjusted to 2.5 mgm. %. The results (table 1) lend support to the hypothesis. Preparations having pressor-oxytocic ratios ranging from 1:15 to 3.5:1 exhibited the same oxytocic potencies by all three methods. When the ratio exceeded 3.5:1 the chicken method and

the uterine method using a magnesium concentration of 2.5 mgm. % agreed, both giving higher values than the official method. This experiment confirms the conclusion of Fraser that "the degree of oxytocic activity observed in an assay depends on the magnesium concentration of the uterine bath, when the ratio between oxytocic and pressor activities of the standard preparation is different from that of the unknown preparation." The experiment shows further, however, that the magnesium concentration of the bath does not affect assay results unless the pressor-oxytocic ratio exceeds 3.5:1.

In order to adduce further evidence that the serum magnesium level was the factor responsible for the high chicken assay values, an attempt was made to potentiate the depressor response to pituitary preparations by the intravenous injection of magnesium immediately preceding the injection of the pituitary.

TABLE 1

*Comparison of results obtained by the official, chicken, and high magnesium uterine methods of oxytocic assay*

PITUITARY SOLUTIONS	PRESSOR OXYTOCIC RATIO	CALCULATED VALUES* PER CENT U. S. P. STANDARD	OFFICIAL METHOD PER CENT U. S. P. STANDARD	CHICKEN METHOD PER CENT U. S. P. STANDARD	HIGH Mg UTER- INE METHOD PER CENT U. S. P. STANDARD
Pitocin .. . . .	1:15		11,000	11,000	11,000
U. S. P. posterior pituitary. . . .	1.1		100	100	100
High P:O 1 . . .	3.5:1	57	57	56	53
High P:O 2 . . .	6.3:1	64	64	50	50
High P:O 3 . . .	9.0:1	75	75	100	100
High P:O 4 . . .	15:1	100	100	250	250
High P:O 5 . . . .	20:1	75	75	222	203
High P:O 6 . . . .	25:1	60	60	240	216
Pitressin .. . .	30:1		150	675	675

\* Calculated on the basis of the official assay values of the pitocin and pitressin from which these solutions were prepared.

Such an experiment is shown in fig. 3. An injection of pitressin (PR) was made. Several minutes later 2.0 cc. of 0.9 % sodium chloride solution (PSS) were injected as a control; this was followed immediately by the same dose of pitressin as was given previously. The pitressin elicited the same depressor response as before. After the blood pressure had returned to normal, 2.0 cc. of an approximately isotonic magnesium chloride solution, containing 5 mgm. of magnesium, were injected and the same dose of pitressin repeated. The depressor response was then much greater than with pitressin alone. The second tracing in fig. 3 illustrates the same fact. The potentiating effect of the magnesium disappeared after 20 to 25 minutes; serum magnesium determinations showed the level to be only slightly elevated after 20 minutes had elapsed and to be approximately normal 25 to 30 minutes after the injection. In order to estimate the magnitude of this potentiating effect, the experiment illustrated in fig. 4 was carried out. An injection of pitressin (PR) caused a blood pressure

fall of 40 mm. However, following the intravenous injection of 5.0 mgm. of magnesium one half the previous dose of pitressin elicited the same fall of blood pressure. Thus the magnesium increased the sensitivity to the pitressin by 100%. Similar experiments showed that the avian depressor responses to U.S.P. standard posterior pituitary solution and pitocin were not significantly affected by the injection of magnesium. This latter finding is what would be expected in view of the report of Fraser that 10 mgm. % anhydrous magnesium chloride (approximately 2.5 mgm. % magnesium), a value which closely approaches the

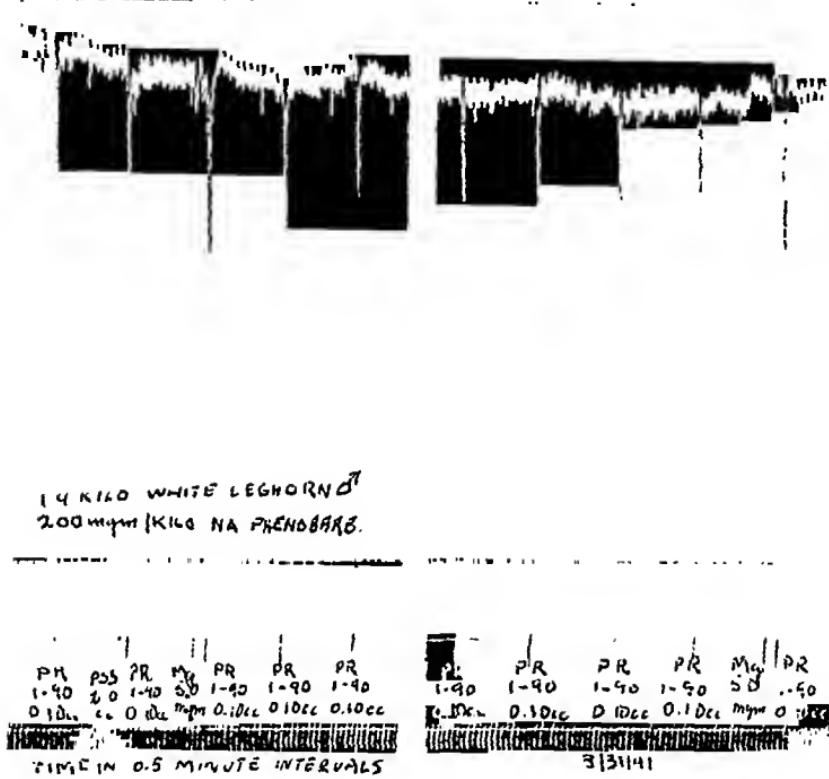


FIG. 3. TRACINGS ILLUSTRATING THE POTENTIATION OF THE AVIAN DEPRESSOR RESPONSE TO PITUITARY PREPARATIONS BY THE INTRAVENOUS INJECTION OF MAGNESIUM

normal serum magnesium level of the chicken, is the level at which postlobin-O exerts its maximum effect on the isolated guinea pig uterus.

In a few preliminary experiments the effect of the intravenous injection of potassium, sodium, calcium, zinc, and cadmium compounds on the sensitivity of the depressor response to subsequent injections of pituitary preparations was studied. None of the ions exerted any appreciable influence on the response.

It was noted by Coon that when a series of small doses of pituitary are injected over a period of time, the depressor sensitivity may decrease to such an

extent that five to eight times the original dose must be injected in order to elicit a response equal to the original one. Similarly, the administration of 25 units of pitocin in one dose will abolish the depressor response to subsequent smaller doses. Since an increase in serum magnesium increases the sensitivity, it was thought possible that the decrease in sensitivity after large doses of pituitary preparations might be due to a fall in the level of the serum magnesium. An experiment was carried out in which 0.2 cc. of a 1-10 dilution of U.S.P. standard solution was injected at three to five minute intervals until the dose no

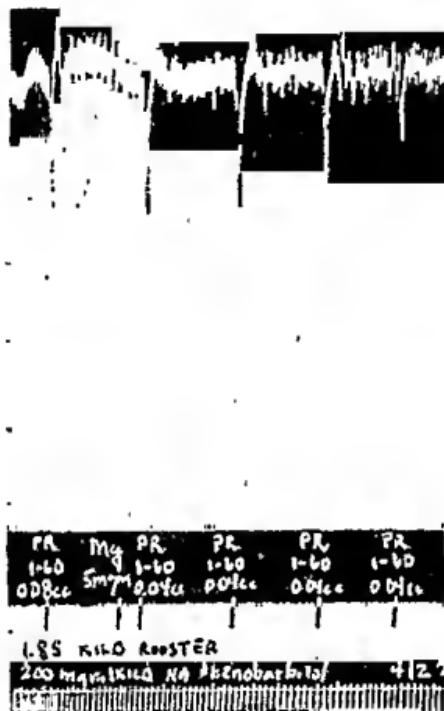


FIG. 4. TRACING ILLUSTRATING A 100 PER CENT POTENTIATION OF THE AVIAN DEPRESSOR RESPONSE TO PITRESSIN BY THE INTRAVENOUS INJECTION OF MAGNESIUM

longer elicited any response. Serum magnesium determinations showed that the loss of sensitivity was not accompanied by any significant variation of the serum magnesium level. Similarly, a massive dose of pitocin (25 U.S.P. units), which abolished the response to subsequent smaller doses, did not change the serum magnesium.

DISCUSSION. The chief disadvantage of the chicken method is the fact that it gives higher values for preparations with high pressor-oxytocic ratios than the official method. This discrepancy is shown to be related to the fact that the serum magnesium level of the chicken is higher than the magnesium titer of the

official Locke-Ringer solution, which contains only a trace of this ion. This conclusion is justified because when the magnesium concentration of the official Locke-Ringer solution is raised to 2.5 mgm. %, the approximate serum magnesium level of the chicken, the uterine and chicken methods give oxytocic values which agree for the various types of posterior pituitary preparations studied, including those with high pressor-oxytocic ratios. The question now arises as to whether the assays obtained for preparations with high pressor-oxytoic ratios by means of the official method are valid, or whether the higher values, obtained by the chicken method and the uterine method using Locke-Ringer solution with 2.5 mgm. % magnesium, are the more nearly correct. Such preparations are not used for their oxytocic activity, but if any specific oxytocic activity is stated to be present it should be that which the preparation will probably exert when used in man. It was demonstrated by Fraser and confirmed in the present investigation that the action of preparations with high pressor-oxytocic ratios on the isolated uterus of the guinea pig depends on the concentration of magnesium in the medium bathing the muscle. That this relationship also holds true for the vascular musculature of the chicken *in situ* is shown by the fact that raising the serum magnesium level increases the sensitivity of the depressor response to subsequent doses of high pressor-oxytocic preparations. The serum magnesium level of man is 2.7 mgm.  $\pm$  0.3 mgm. %, according to Greenberg and his associates (18). It appears probable, therefore, that when preparations with high pressor-oxytocic ratios are used in man they exert an oxytocic activity which approaches more closely the potency indicated by the chicken method and the uterine method using high magnesium Locke-Ringer solution than that indicated by the official method.

As reported by Coon and verified in the present investigation, the chicken method possesses many practical advantages over the official uterine assay. These advantages include relative simplicity and economy of time and funds. It is felt in this laboratory that this new method should receive consideration for official recognition.

#### SUMMARY

1. The finding of Coon that the chicken method yields higher oxytocic values than the official method for preparations which have high pressor-oxytocic ratios is confirmed.

2. This discrepancy is explained by the difference between the low magnesium concentration of the official Locke-Ringer solution and the higher magnesium concentration of chicken blood serum. When the magnesium titer of the Locke-Ringer solution used in the official uterine method is raised to 2.5 mgm. %, the approximate serum magnesium level of the chicken, the discrepancy disappears.

3. The conclusion of Fraser that "the degree of oxytocic activity observed in an assay depends on the magnesium concentration of the uterine bath, when the ratio between the oxytocic and pressor activities of the standard preparation is different from that of the unknown preparation" is confirmed and extended.

4. Raising the serum magnesium level of the chicken by the intravenous in-

jection of magnesium chloride increases the sensitivity of the depressor response to preparations having high pressor-oxytocic ratios but not to preparations having a ratio of one or less. The effect wears off rapidly, disappearing entirely at approximately the same time that the serum magnesium level returns to normal.

5. The oxytocic activity which a preparation with a high pressor-oxytocic ratio will exert when used in man is probably indicated more accurately by the chicken method and the uterine method, using a Locke-Ringer solution with a magnesium concentration of 2.5 mgm. %, than by the official uterine method.

6. Neither the progressive decrease in sensitivity of the avian depressor response, which accompanies the injection of a series of small doses of posterior pituitary preparations, nor the abolition of the response after a massive dose of pitocin is due to a fall in the blood serum magnesium level.

7. The claims of Coon as to the many practical advantages of the chicken method are confirmed. The technique should receive consideration for recognition as an official method of assay.

The author wishes to express his appreciation to Doctors E. M. K. Geiling and J. M. Coon for their criticisms and advice. He also wishes to thank Miss Elizabeth Webster for her assistance. Acknowledgment is made of grants from the Board of Trustees of the United States Pharmacopoeia and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago, which aided the investigation.

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# THE GASTRO-INTESTINAL ABSORPTION OF LANATOSIDE C

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Lanatoside C is a crystalline glycoside isolated in chemically pure form from *Digitalis lanata*. It possesses the therapeutic properties of the cardioactive glycosides and has been used principally in intravenous therapy where its rapid action gives it strophantidin-like qualities (1, 2). However, it is well tolerated orally and being a pure substance with a uniform potency its use for oral medication seems likely to become more extensive. This paper reports data on the absorption of Lanatoside C from the gastro-intestinal tract of cats, comparing it with the absorption of the tincture of digitalis.

**EXPERIMENTAL PROCEDURES.** The extent and rate of absorption of digitalis glycosides from the gastro-intestinal tract is commonly determined experimentally by comparing the Lethal Doses, determined by intravenous infusion, with and without previous gastro-intestinal administration of the drug. This procedure can be termed the determination of "essential absorption" and is based on the assumption that death will always occur when a Lethal Dose has accumulated in the animal regardless of the route of administration. As usually carried out, a quantity of the drug is placed in a ligated section of the gastro-intestinal tract, and, after a given period of time, administration by intravenous infusion at a uniform rate and continued until death. The amount administered in this way is then subtracted from the amount which produces death by the same method of intravenous infusion in a control animal and the difference is assumed to be the amount absorbed from the gastro-intestinal tract. This procedure has been used by Hatcher (3), Haskell, McCants and Gardner (4), Nyary (5) and Svec (6) to study the rate of absorption of various digitalis preparations. As ordinarily used this procedure is subject to some objection because absorption from the ligated section of the gastro-intestinal tract is still going on when the intravenous infusion is made and hence the experimental procedure is not identical with the control.

Another method depends upon the determination of the residual drug in the tract after the period of absorption. Using colorimetric methods Gottlieb and Ogawa (7) and Van-Liere and Emerson (8) have studied the absorption of various preparations and glycosides. The colorimetric methods, however, are not very satisfactory. No reports of attempts to estimate the residual glycosides biologically were found.

The procedures used in the experiments reported here make use of both of these methods. Essential absorption was determined according to the general procedure described above but at the end of the period allowed for absorption from the ligated section the latter was removed to prevent further absorption during the period of intravenous infusion. This also made it possible to subject the contents of the excised section to a simple biological assay thus securing results from two procedures in the same experiment.

**Determination of Essential Absorption.** The cat was anesthetized by the administration of 35 mgm. of sodium pentobarbital intraperitoneally. A tracheal cannula was inserted, the viscera were exposed and the section of the gastro-intestinal tract to be studied was isolated by means of two ligatures which were placed, without disturbing the mesenteric blood vessels, at each end of the section. The solution of the drug was injected into the section and the abdominal incision temporarily closed. A short time before the end of the period allowed for absorption a cannula was inserted into the carotid artery to record blood

pressure. A venous cannula was inserted into the femoral vein and attached to a burette arranged to deliver a solution intravenously at 33°C. At the end of the period of absorption the blood vessels supplying the ligated section were tied off and the entire section removed by cutting between the double ligatures previously placed at each end of the section. The iotrofroponous infusion was then begun. If Lanatoside C was used 0.0015% solution was infused at the rate of 0.5 cc. per kgm. per minute. The stock solution of this drug was prepared by dissolving 0.010 grams of Lanatoside C in 5.00 grams of 95% alcohol with slight heat and making this up to 50 cc. with distilled water. This solution then contained 12% alcohol by volume. If Tincture of Digitalis was used the Tincture of Digitalis U.S.P. XI was diluted with distilled water to contain the equivalent of 0.005 grams of the dried drug per cc. and was infused at the rate of 0.5 cc. per kgm. per minute. The endpoint was taken at the time the blood pressure fell to zero.

The determination of the Lethal Dose by intravenous infusion in the control experiments was carefully made with all of the conditions of anesthesia and surgery described above carried out. About 10 cc. of physiological saline was injected into the section instead of the drug solution. This determination of the Lethal Dose by slow intravenous infusion is the same procedure used for the biological assay of digitalis on cats but with the difference that it was here determined under the anesthesia, surgery and other conditions used in an actual absorption experiment.

In these control experiments the quantity of Lanatoside required to produce death was 0.36 mgm. per kgm. (mean of 6 cats, range 0.21 to 0.49). Moe and Visscher (9) found the Lethal Dose to be 0.39 mgm. per kgm. (mean of 7 cats, range 0.29 to 0.44). Similar control experiments gave a Lethal Dose of Tincture of Digitalis U.S.P. XI of 0.11 gram of the dried drug per kgm. (mean of 6 cats, range 0.09 to 0.13). The Lethal Dose of the Tincture of Digitalis U.S.P. XI is generally assumed to be 0.10 gram per kgm. These values are not significantly different although ours were determined under special conditions.

*Assay of the Gastro-Intestinal Residue for Glycosides.* The section of the gastro-intestinal tract which had been removed at the end of the period of absorption as described above was opened and the contents drained into a beaker. The entire section was then hashed in a meat grinder and washed repeatedly with 95% alcohol. Preliminary experiments in which known amounts of glycoside had been placed in the lumen of the intestine showed this to be necessary because the glycoside apparently clings tenaciously to the walls of the intestine. The washings and contents were combined, filtered, and evaporated at 58°C. to a convenient volume. Three volumes of alcohol were added, the mixture evaporated to about 25 cc., filtered and then evaporated to dryness in a small beaker. The residue was then dissolved in a known quantity of alcohol, and water added to make the resulting solution contain 12% alcohol.

The solution thus prepared was biologically assayed by the method described by Hoyer (10). This method involves the determination of the time required for a given concentration of the drug to stop a frog's heart *in situ*. The solution is held in contact with the heart by a small glass chamber sutured to place and the heart contractions are recorded on a kymograph by means of a lever. Several kymographic setups expedited these determinations. A time-concentration curve such as described by Trevan (11) was first prepared by plotting the time required to produce stoppage against the concentration of the solution by using a series of known concentrations. The mean of five determinations was used for each point. The extremes of the curve for Lanatoside C showed stoppage in 15 minutes with 0.20 mgm. per cc. and stoppage in 30 minutes with concentrations of 0.13 mgm. per cc. The curve for Tincture of Digitalis U.S.P. XI showed stoppage in 8 minutes with the equivalent of 0.10 gram of the dried drug and stoppage in 18 minutes with a concentration equivalent to 0.03 gram of the dried drug per cc. of the solution applied to the heart. To determine the concentration of glycoside in an unknown solution two or three exploratory runs were made to determine the approximate concentration which would produce stoppage within a period of time that would fall within the previously determined curve, then five determinations were made and the mean of these values used to determine the concentrations of the

unknown from the curve. From this the residual amount of Lanatoside C or digitalis in the section of the gastro-intestinal tract was then calculated.

There is always a considerable number of variables which influence the quantity of a drug absorbed from the gastro-intestinal tract and its rate of absorption. In order to produce as uniform a set of conditions as possible, the cats were starved for 24 hours before the determinations, the dose of the drug placed in the isolated section of the tract was always approximately five times the intravenous Lethal Dose and the volume injected into the ligated section was always the same per kilogram; 10 cc. of the stock solution of Lanatoside

TABLE 1

*The absorption of Lanatoside C from portions of the gastro-intestinal tract of cats*

In each case a solution of Lanatoside C containing 2.0 mgm. per kgm. (approximately 5 times the lethal dose determined by intravenous infusion) was placed in the ligated section and absorption allowed to take place for one hour

PORTION OF GASTRO-INTESTINAL TRACT	CAT NUMBER	LETHAL DOSE AFTER ONE-HOUR ABSORPTION PERIOD	CALCULATED ESSENTIAL ABSORPTION*	BIOLOGICAL ASSAY OF RESIDUAL GLYCOSIDE IN THE INTESTINE	
				Amount recovered	Apparent absorption
Stomach		mgm./kgm.	mgm./kgm.	mgm./kgm.	mgm./kgm.
	19	0.31	0.05	1.76	0.24
	20	0.24	0.12	1.51	0.49
	21	0.23	0.13	1.47	0.53
	27	0.28	0.08	1.62	0.38
Intestine†	28	0.26	0.10	1.52	0.48
	17	0.00	0.36 (in 34 min.)	1.19	0.81
	18	0.00	0.36 (in 42 min.)	1.14	0.86
	16	0.00	0.36 (in 60 min.)	0.97	1.03
	25	0.15	0.21	1.32	0.68
	26	0.01	0.35	1.64	0.36
	32	0.08	0.28	1.28	0.72
Colon	33	0.04	0.32	1.20	0.80
	22	0.25	0.11	1.86	0.14
	23	0.22	0.14	1.63	0.37
	24	0.26	0.10	1.87	0.13
	37	0.28	0.08	1.84	0.16
	38	0.24	0.12	1.70	0.30

\* The mean intravenous lethal dose determined in the control experiments (0.36 mgm. per kgm.) minus the lethal dose determined after gastro-intestinal absorption was allowed to take place.

† Jejunum and ileum.

C described above or 6 cc. of the Tinetur of Digitalis U.S.P. XI diluted to 10 cc. with distilled water. The alcohol content differed being 12% in the case of Lanatoside C solution and about 40% in the case of Diluted Tinetur of Digitalis.

**EXPERIMENTAL RESULTS. The Site of Absorption.** Absorption was studied from the following sections of the gastro-intestinal tract: the stomach, the intestine and the colon. The section referred to as the intestine consists of the jejunum and the ileum only, the anterior ligatures being placed at the point of attachment of the tail of the pancreas to the intestine. This was done in order

to avoid the delay which the required surgery would have made necessary at the time of excision had the duodenum been included. The posterior ligatures were placed at the ileocecal junction. The solution of the drug was then introduced into the section by means of a hypodermic needle and syringe. The period of absorption in each case was one hour. Table 1 shows the quantities of Lanatoside C absorbed from these three portions of the gastro-intestinal tract as determined by the method of essential absorption and by the biological estimation of the residual glycoside. Cats 17, 18, and 16 died before the intravenous

TABLE 2

*The absorption of Digitalis from portions of the gastro-intestinal tract of cats*

In each case a diluted Tincture of Digitalis, U. S. P. XI, containing the equivalent of 0.60 grams of dried drug per kgm. (approximately 5 times the lethal dose by intravenous infusion) was placed in the ligated section and absorption allowed to take place for one hour.

PORTION OF GASTRO-INTESTINAL TRACT	CAT NUMBER	LETAL DOSE AFTER ONE-HOUR ABSORPTION PERIOD grams/kgm.	CALCULATED ESSENTIAL ABSORPTION* grams/kgm.	BIOLOGICAL ASSAY OF RESIDUAL GLYCOSIDES IN THE INTESTINE	
				Amount recovered grams/kgm.	Apparent absorption grams/kgm.
Stomach	11	0.03	0.03	0.43	0.17
	12	0.06	0.05	0.47	0.13
	39	0.03	0.03	0.45	0.15
	40	0.06	0.05	0.52	0.08
	41	0.07	0.04	0.48	0.12
Intestine	7	0.06	0.05	0.53	0.07
	8	0.05	0.06	0.52	0.08
	29	0.05	0.06	0.52	0.08
	30	0.04	0.07	0.50	0.10
	31	0.03	0.08	0.48	0.12
Colon	9	0.11	0.00	0.58	0.02
	10	0.10	0.01	0.58	0.02
	34	0.07	0.04	0.54	0.06
	35	0.08	0.03	0.56	0.04
	36	0.10	0.01	0.58	0.02

\* The mean intravenous lethal dose determined in control experiments (0.11 grams per kgm.) minus the lethal dose determined after gastro-intestinal absorption had been allowed to take place.

infusion was begun, which indicates that sufficient of the Lanatoside was absorbed from their intestine to produce death in an hour or less. Cat 26 required only a very little additional drug by the infusion route to produce death, while cat 25 had absorbed almost two-thirds of a Lethal Dose. This would indicate that approximately one Lethal Dose is absorbed in a hour from the ligated portion of the intestine under these conditions; about one-third of a Lethal Dose is absorbed from the ligated colon and somewhat less than one-third of a Lethal Dose is absorbed from the ligated stomach.

Under the same conditions, as shown in table 2, allowing one hour for absorp-

tion with 5 times the Lethal Dose in the ligated section, the absorption of Tincture of Digitalis U.S.P. XI was slower than the absorption of Lanatoside C. In one hour about half of a Lethal Dose was absorbed from the ileum and jejunum, less than one-tenth Lethal Dose from the colon and a third to a half from the stomach.

*The Rate of Absorption.* In the foregoing experiments the time allowed for absorption was maintained uniformly at one hour. In these experiments the time was varied and the amount of absorption which had taken place from the

TABLE 3

*The rate of absorption of Lanatoside C and Tincture of Digitalis, U. S. P. XI, from the intestine of cats*

A solution of 2.0 mgm. per kgm. of Lanatoside C of a diluted solution of the tincture equivalent to 0.60 grams of digitalis per kgm. was introduced into the ligated intestine

DRUG	CAT NUMBER	TIME ALLOWED FOR ABSORPTION	LETHAL DOSE DEATH, AFTER PERIOD OF ABSORPTION	CALCULATED ESSENTIAL ABSORPTION*	BIOLOGICAL ASSAY OF RESIDUAL GLYCOSIDES IN THE INTESTINE	
					Amount recovered	Apparent absorption
Lanatoside C	42	5	0.31	0.05	1.93	0.07
	43	10	0.14	0.22	1.74	0.26
	56	10	0.23	0.13	1.62	0.38
	52	20	0.20	0.16	1.68	0.32
	44	30	0.09	0.27	1.46	0.54
	57	30	0.04	0.32	1.58	0.42
	51	40	0.12	0.24	1.50	0.50
	45	50	0.03	0.33	1.48	0.52
Tr. Digitalis U.S.P. XI			gm./kgm.	gm./kgm.	gm./kgm.	gm./kgm.
	46	5	0.10	0.01	0.56	0.04
	54	20	0.09	0.02	0.53	0.07
	53	30	0.07	0.04	0.52	0.08
	47	90	0.05	0.06	0.44	0.16
	55	90	0.04	0.07	0.47	0.13
	48	120	0.04	0.07	0.45	0.15
	58	120	0.02	0.09	0.46	0.14
	50	150	0.01	0.10	0.40	0.20
	59	150	0.00	0.11	0.42	0.18
	49	180	0.01	0.10	0.41	0.19

\* See footnotes to tables 1 and 2.

intestine at the end of various time intervals was determined. These results are shown in table 3 in which the absorption is shown for Lanatoside C for periods from 5 minutes to 50 minutes, and for Tincture of Digitalis from 5 minutes to three hours. The absorption at the end of an hour period can be seen in table 1. These results indicate that the absorption of Lanatoside C is about three times as rapid as the Tincture of Digitalis.

*DISCUSSION.* The use of a biological assay for the determination of the amount of glycoside remaining in the gastro-intestinal tract after a period of absorption as a means of checking the results of the essential absorption pro-

dure gives an interesting correlation. The results lead to the same conclusions reached by the essential absorption method, but the figures arrived at by subtracting the amount of glycoside recovered from the amount placed in the ligated section are much higher and would seem to indicate that more than a Letal Dose has been absorbed. We have referred to this value as "Apparent Absorption" because there is actually no justification for assuming that the difference between the amount put into the section and the amount recovered is entirely absorbed. While this discrepancy may be due in part to the inadequacy of the bioassay method used to estimate the residual glycoside in the excised section, it is very likely that a portion of the glycoside has been destroyed by the digestive enzymes. Svec (12), and Brucke (13) have both shown that gastric juice has the property of decreasing the activity of digitalis glycosides. Evidence for the destruction of the digitalis glycosides in the intestine is not so conclusive but Holste (14) found that diastase, pancreatin and intestinal juice each decreased the potency of the glycosides, and Hale (15) obtained similar results from artificial pancreatic juice. No reports were found on destruction of the glycosides in the colon.

#### CONCLUSIONS

The greatest absorption of Lanatoside C and Tincture of Digitalis U.S.P. XI takes place in the intestine, but quantities are also absorbed from the colon and stomach. Lanatoside C is absorbed more rapidly from the intestine than the Tincture of Digitalis.

Differences between the results from analysis of residual glycosides in portions of the gastro-intestinal tract and the determination of essential absorption indicate that partial destruction or inactivation of both Lanatoside C and the glycosides of the Tincture of Digitalis U.S.P. XI take place in the lumen of the gastro-intestinal tract.

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## STUDIES ON SULFONAMIDE-RESISTANT ORGANISMS: II. COMPARATIVE DEVELOPMENT OF RESISTANCE TO DIFFERENT SULFONAMIDES BY PNEUMOCOCCI

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Experimental and clinical studies (1-13) have shown that pneumococci may become resistant to the action of a sulfonamide. It is of practical importance to know: (1) the capacity of different strains of pneumococcus to develop resistance; (2) the relative ease with which such resistance is acquired to different sulfonamides; and (3) whether a strain which has acquired resistance to one sulfonamide is also resistant to other drugs of this group.

The present report concerns a study of these problems. It should be pointed out, however, that some experiments (7, 12, 14) have already indicated that strains of pneumococcus which have become resistant to either sulfapyridine, sulfathiazole or sulfamethylthiazole are resistant to all three of these drugs.

**METHODS.** Three strains of pneumococcus (type I, strain McGovern; type II, strain CH; and type III, strain CHA) were used in this study. These strains have been studied in this laboratory over a period of three years; consequently, their cultural characteristics and *in vivo* and *in vitro* responses to several sulfonamides have been well established.

Each of the above strains was made resistant to sulfanilamide, sulfapyridine and sulfathiazole by serial passage through media containing increasing quantities of these drugs. With two exceptions the method used was the same as that described previously (9). In the present work visual measurements of growth were substituted for actual growth curves, and transfers to successive lots of drug broth were made at shorter intervals than in the preceding study. The basal medium employed was a beef heart infusion broth, preparation of which has been described previously (15). Concentrated solutions of sulfanilamide (320 mgm. %), sulfapyridine (150 mgm. %), and sulfathiazole (150 mgm. %) were prepared by dissolving the powdered drugs in this basal medium. These sulfonamide solutions were diluted with basal medium so that the final concentrations of drug, after addition of all constituents, including inoculum, were 2.5, 5, 10, 20, 40, 80, and 135 mgm. % of sulfapyridine or sulfathiazole, and 5, 10, 20, 40, 80, 160, and 290 mgm. % of sulfanilamide. The various sulfonamide solutions were tubed in 9 cc. quantities and sterilized by autoclaving. Two % rabbit blood was added just before use.

At the beginning of the investigation the stock strains, which had been passed repeatedly through mice, were grown 12 to 14 hours in beef infusion broth enriched with blood. These cultures were diluted serially in basal medium and 1 cc. of a  $10^{-4}$  dilution was added to each of a series of tubes containing the lower concentrations of drug—giving a final inoculum of 1000 to 5000 pneumococci per cc. Growth (i.e. turbidity and change in color of the red cells (16)) was noted after 24 hours incubation at 37.5°C. At that time a transfer was made from the highest concentration of drug broth showing visible growth into control blood broth. This control broth transfer was incubated 12 to 14 hours, diluted serially and 1 cc. of a  $10^{-4}$  dilution was added to each of another series of drug broth tubes. This procedure was repeated,<sup>1</sup> the drug concentrations being increased in each succeeding experi-

<sup>1</sup> In most instances the organisms were incubated in drug broth for 24 hours as in the original experiment. In a few cases, however, incubation in drug broth was continued for 48 hours before subcultures were made.

ment, until rapid growth occurred in one of the highest concentrations of the sulfonamide used.

When a strain had become highly resistant to a given sulfonamide, its response to this drug was compared with its response to each of the other two drugs and to sulfadiazine. This was accomplished by inoculating series of tubes containing various concentrations of the different drugs, prepared as described above, and determining the highest concentration of each sulfonamide permitting visible growth at the end of 24 and 48 hours incubation.

In order to determine whether repeated passage in the basal medium affected sulfonamide sensitivity, the parent strains were passed serially through control broth each time the "resistant" strains were transferred in drug broth. At the end of the series of transfers, these control strains were tested against the various sulfonamides in the same manner as were the resistant organisms.

**RESULTS.** The development of resistance to the sulfonamides by the three strains of pneumococcus has been summarized in figure 1. The points plotted are the highest concentrations of drug which allowed visible growth at a given transfer.

It is evident from the initial response of each of the strains that the parent organisms were sensitive to all three drugs. There was some variation, however, in the response of the different strains to the same drug, type II CH appearing to be the most sensitive and type III CHA the most resistant. There was also a difference in the response of the same strain to the different drugs, each organism being most sensitive to sulfathiazole and least sensitive to sulfanilamide.<sup>2</sup>

When the organisms were passed serially through drug broth there was a progressive increase in resistance until finally each strain was able to grow in very high concentrations of the sulfonamides. There were some irregularities in this process, as indicated by the fact that at certain points the resistance remained stationary for two or more transfers. It seems probable, however, that such plateaus might have been avoided if smaller increments of drug had been used.

In the early stages of the experiment there were characteristic differences in the rates at which the three strains became sulfonamide resistant. These differences were not as clear cut in the work with sulfanilamide, to which each strain acquired resistance rapidly, as in that with sulfapyridine and sulfathiazole. In the experiments with the latter drugs the type III CHA strain acquired resistance most rapidly and the type II CH strain most slowly. In view of the rapidity with which type III CHA became resistant, it is of interest that the final degree of resistance attained by this organism was not as great as that of the other two strains.

The order in which resistance was developed to the different drugs was the same with each of the three strains. Sulfanilamide resistance was developed most rapidly, sulfapyridine resistance next in order, and sulfathiazole resistance most slowly.

After resistance to one sulfonamide had been established, the response of these resistant strains to each of the other two sulfonamides and to sulfadiazine

<sup>2</sup> Although the data recorded for the type I and III strains indicate that sulfathiazole and sulfapyridine were of equal effectiveness after 24 hours incubation, observations made at earlier periods show clearly that sulfathiazole inhibited growth more than did sulfapyridine.

was determined. The results of these tests are listed in table 1, together with the reactions of the strains which had undergone serial passage through the control medium.

It should be noted that each of the three control strains grew only in low concentrations of all the drugs, just as had the parent organisms (figure 1). On the other hand, the resistant strains grew in very high concentrations, not only of the drug to which each organism had been made resistant but also of all the other sulfonamides tested. All the resistant strains of type I McGovern

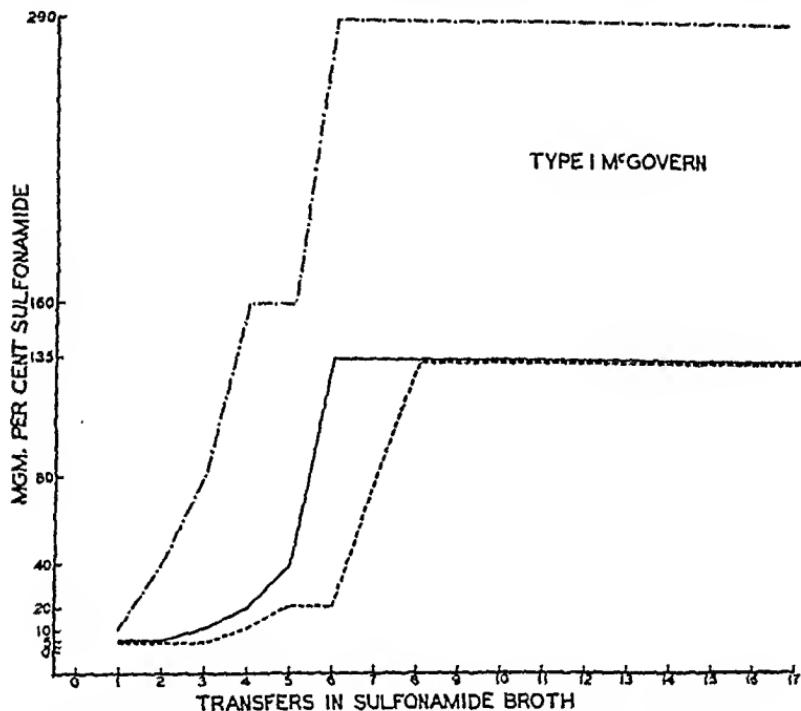


FIG. 1. DEVELOPMENT OF RESISTANCE TO DIFFERENT SULFONAMIDES

Development of sulfanilamide resistance -----  
 Development of sulfapyridine resistance —————  
 Development of sulfathiazole resistance - - - - -

and type II CH grew in the highest concentrations of each of the drugs at 48 hours; many had grown in the highest concentrations as early as 24 hours. The type III CHA strains grew more slowly than either the type I McGovern or type II CH organisms. As a consequence of this, growth of the type III CHA strains in the higher drug concentrations did not always occur in 24 hours. Such growth did occur, however, within 48 hours.

**COMMENT.** It should be pointed out that the problem of sulfonamide resistance merits consideration whenever therapy is prolonged, for both experimental (9) and clinical observations (17) have shown that under this condition

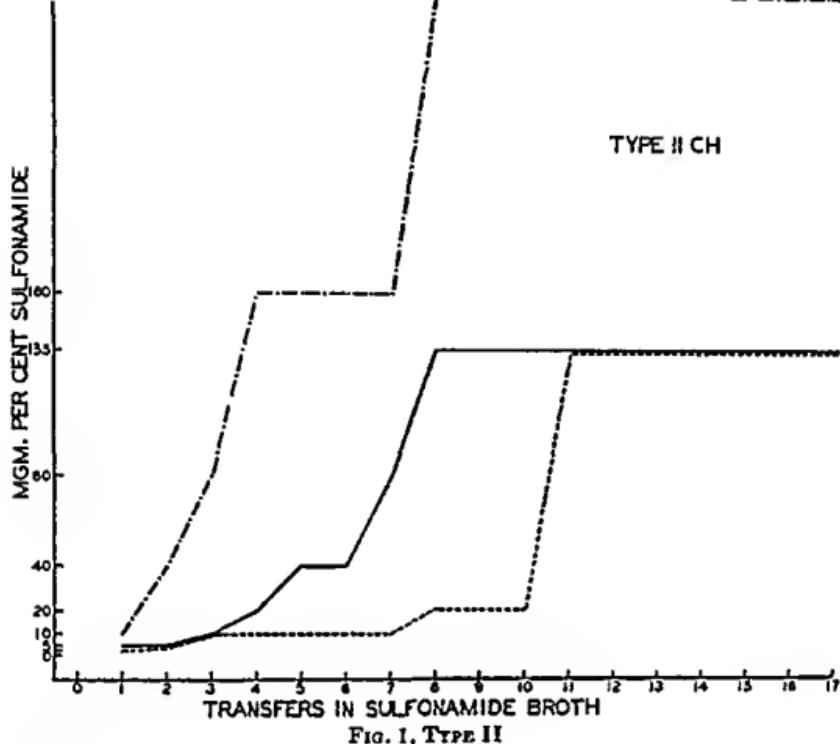


Fig. 1, TYPE II

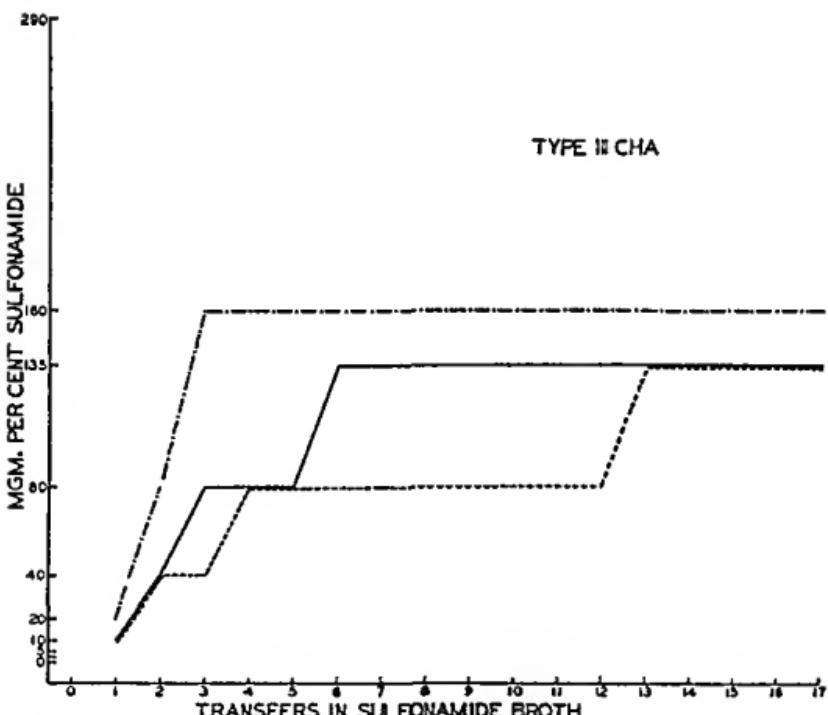


Fig. 1, TYPE III

resistance is most likely to develop. Although the present study, like most other work on sulfonamide resistance, has been concerned with the response of pneumococci, the general conclusions from this work may well have application to the reactions of other organisms. Actually, the problem of sulfonamide resistance may be of greater practical importance in infections caused by other bacteria than it is in pneumococcal infections, which are usually of short duration and respond promptly to treatment.

From the data presented here it is apparent that strains of pneumococcus differ in their abilities to develop sulfonamide resistance. There is a suggestion

TABLE I  
*Response of resistant pneumococci to various sulfonamides*

STRAIN	INOCULUM NO. OF ORGANISMS PER CC. CULTURE	MG.M. PER CENT OF DRUG PERMITTING VISIBLE GROWTH*							
		After 24 hrs. incubation				After 48 hrs. incubation			
		ST	SP	SD	SA	ST	SP	SD	SA
<i>Type I McGovern</i>									
Control strain.....	3200	C†	C	10	20	C	5	10	20
Sulfathiazole resistant.....	279	135	135	135	290	135	135	135	290
Sulfapyridine resistant.....	3600	135	135	135	290	135	135	135	290
Sulfanilamide resistant.....	2500	80	135	135	290	135	135	135	290
<i>Type II CH</i>									
Control strain.....	1900	C	C	5	C	C	C	5	C
Sulfathiazole resistant.....	1900	80	80	135	290	135	135	135	290
Sulfapyridine resistant.....	1300	135	135	135	290	135	135	135	290
Sulfanilamide resistant.....	3000	135	135	135	290	135	135	135	290
<i>Type III CHA</i>									
Control strain.....	2300	C	5	5	10	C	10	10	20
Sulfathiazole resistant.....	1240	20	40	135	80	80	135	135	290
Sulfapyridine resistant.....	1500	80	135	135	80	80	135	135	290
Sulfanilamide resistant.....	1800	20	40	80	80	80	80	135	160

\* The concentrations of drug used were 0, 5, 10, 20, 40, 80, and 135 mgm. % of sulfathiazole, sulfapyridine and sulfadiazine, and 0, 10, 20, 40, 80, 160, and 290 mgm. % of sulfanilamide.

ST = sulfathiazole; SP = sulfapyridine; SD = sulfadiazine; SA = sulfanilamide.

† C indicates that growth occurred in the control broth but not in broth containing the lowest concentration of drug.

that this capacity for developing resistance is related to the initial sensitivity of the organisms, inasmuch as the type II CH strain, initially highly sensitive to sulfonamides, acquired resistance with greater difficulty than the type III CHA organism which was naturally somewhat resistant. If this suggestion is correct, then one would expect that organisms like staphylococci, which naturally are relatively sulfonamide-insensitive, would acquire resistance with great ease. This may be one of the factors responsible for the numerous failures of sulfonamide therapy in staphylococcal infections.

The present report has also shown that a given strain of pneumococcus develops resistance to the various drugs at different rates. Resistance to sulfanil-

amide was developed most rapidly and to sulfathiazole most slowly. This indicates that the ease of developing resistance varies inversely with the activity of the sulfonamide, since in the medium used sulfanilamide was the least effective and sulfathiazole the most effective drug. Similar observations have been made in studies on *E. coli* (18). These findings are a further argument for knowing which drug has the greatest activity against a specific organism.

Finally, the data have shown that once pneumococci have developed resistance to either sulfanilamide, sulfapyridine, or sulfathiazole, they are also resistant to each of the other two drugs and to sulfadiazine. This finding is in harmony with those of previous investigators (7, 12) and has also been shown to be true *in vivo* (14, 19). Since the activities of the different sulfonamides against pneumococci are relatively similar (16, 20, 21), it may be concluded that there would be little benefit in changing therapeutic agents in pneumococcal infections once resistance was established. However, where there are striking differences in the activities of the drugs, as in the effects of sulfanilamide and sulfathiazole on gonococci (8) and *E. coli* (18, 22), the more active drug might still be effective against organisms that had acquired resistance to the less active sulfonamide. This is borne out by the work of Carpenter *et al.* (23) in which sulfathiazole inhibited the growth of gonococci that had acquired resistance to sulfanilamide.

#### SUMMARY

A study of some of the factors involved in the development of sulfonamide-resistant pneumococci showed the following:

- (1) Strains of pneumococcus differed in the ease with which they developed resistance to a given sulfonamide.
- (2) There was a difference in the rates at which a single strain of pneumococcus developed resistance to the various sulfonamides. In all instances, resistance was developed most rapidly to the least effective drug and most slowly to the most effective drug.
- (3) When a strain of pneumococcus had acquired resistance to one sulfonamide, it was also resistant to all the other sulfonamides tested.

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# A STUDY OF THE COMPARATIVE TOXIC EFFECTS OF MORPHINE ON THE FETAL, NEWBORN AND ADULT RATS<sup>1</sup>

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Various investigators report that newborn animals are less resistant to the toxic effects of morphine than adults of the same species. Gihhs and Bohh (1) noted that adult rats injected hypodermically with morphine are ten times as resistant as newborns. Schlossmann (2) found that the lethal dosage of morphine hydrochloride (LD 50) on intraperitoneal injection of rabbis is 0.15 gm. per kilo for rabbis one week of age, and about 0.5 gm. per kilo for rabbis over three months of age. Eddy (3) using subcutaneous injections also found that adult rabbis are more resistant than newborn rabbits. The problem deserves further investigation, however, since morphine is such an important analgesic that obstetricians continue to use it despite the comparatively high susceptibility of the newborn.

**EXPERIMENTAL.** The relative resistance of newborn and adult rats to morphine was determined by comparing their survival time after intraperitoneal injection of various dosages of morphine sulphate. Maintenance of respiratory activity was taken as the criterion of survival. The mortality of adult rats succumbing at 1, 2, and 12 hours with the range of doses used is presented in table 1. All the adults injected with 0.92 mgm./gm. died within an hour. With doses below 0.84 mgm./gm., some of them recovered.

The average survival time of over 300 newborn rats injected with solutions of 0.01 to 4% morphine sulphate in doses ranging from 0.0005 to 1.32 mgm./gm. is presented in figure 1. The survival time of the newborns lengthened rapidly as the dose was decreased below 0.1 mgm./gm. but did not shorten significantly as the dose was increased above 0.1 mgm./gm. Because their survival time did not continue to decrease with the higher range of doses, the possibility of another mechanism for energy, not depressed by morphine, should be considered.

A comparison of the figures shows that despite the fact that infants succumb to smaller doses than adults, they nevertheless respire longer than adults with large lethal doses. With 0.92 mgm./gm., a dose to which all adults died in less than an hour, newborns survive an average of 3½ hours.

To determine the effects of morphine on the fetus, pregnant mothers at full term were injected with morphine sulphate. The fetuses were removed by Caesarean section immediately after the mother had succumbed to respiratory failure or when she was sacrificed after a period of two hours. The effects were found to differ, depending on the dosage used and the time of survival of the

<sup>1</sup> Aided by a grant from the Albion O. Bernstein Memorial Fund.

mother after injection. The fetuses, removed from 3 mothers dying within 30 minutes after injection of 1.08 mgm./gm., either breathed spontaneously or

TABLE 1

*Effects of intraperitoneal injections of 2% morphine sulfate on the survival time of adult rat*

DOSE IN MG.M./GM. BODY WEIGHT	NUMBER OF RATS INJECTED	SUCCUMBED IN 1 HOUR	SUCCUMBED IN 2 HOURS	SUCCUMBED IN 12 HOURS	RECOVERED
0.22	14	1	1	2	10
0.32	13	3	2	2	6
0.42	15	1	5	4	5
0.52	13	6	2	0	5
0.62	12	6	2	3	1
0.70	12	7	1	1	3
0.76	13	9	0	1	3
0.84	12	11	0	1	0
0.92	12	12	0	0	0

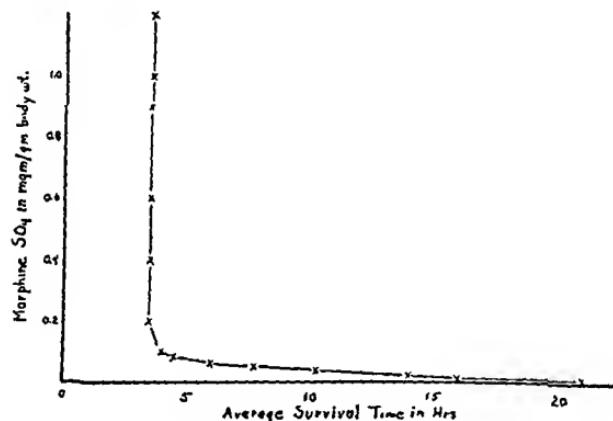


FIG. 1. SURVIVAL TIME OF NEWBORN RATS WITH MORPHINE

TABLE 2

*Comparative survival periods of three groups of newborn rats, the first, injected with morphine and respiring nitrogen; the second, uninjected and respiring nitrogen; and the third, injected with morphine and respiring air*

MORPHINE IN MG.M. PER GM. BODY WEIGHT	AVERAGE SURVIVAL TIME		
	Injected rats in nitrogen	Uninjected controls in nitrogen	Injected controls in air
0.03	min.	min.	hrs.
0.32	51	57	13
0.92	49	58	3½

could be stimulated to respire for a short time. All fetuses removed from a fourth mother surviving this dose for 58 minutes were dead. When fetuses were

removed from 7 mothers injected with 0.42 mgm./gm., only 1 or 2 of the 10-12 members comprising each litter could be stimulated to breathe, but respirations were not maintained for longer than 2 hours. Thus, fetuses appeared to have approximately the same susceptibility to the acute lethal effect of morphine as the adult and did not demonstrate the increased resistance of the newborn.

The effects of morphine on the resistance of the newborn to anoxia were studied. Newborn rats injected with morphine were placed in an atmosphere of undiluted nitrogen to produce anaerobiosis. The duration of respiration in these rats was compared with that of uninjected litter-mate controls respiration nitrogen. Other litter-mate controls, breathing air, were injected with the same doses of morphine. Table 2 shows that the newborns injected with morphine did not live for a significantly shorter time in nitrogen than did the uninjected controls.

Intraperitoneal injections were used for all our procedures, since in previous control experiments on adult rats the slow absorption of 2% morphine sulphate from subcutaneous depots permitted these animals to recover from doses larger than those which are lethal on intraperitoneal injection.

**DISCUSSION.** Our task is to explain why the newborn lives longer than the adult and why the fetus *in utero* does not live so long as the infant *postpartum*. The greater resistance of newborns to the acute morphine depression is probably explained by their greater resistance to anoxia. Previous work in this laboratory has disclosed that newborn rats survive for approximately 60 minutes in an atmosphere of undiluted nitrogen (5). The oxygen present in the lungs is not an important factor for this survival because the oxygen content of the arterial blood of animals becomes minimal a short time after exposure to undiluted nitrogen (5). The newborn is able to survive during this period chiefly because of its anaerobic utilization of carbohydrate (6). The energy for survival is derived by the anaerobic splitting of carbohydrate to lactic acid. Adults also have this anaerobic source of energy available, but the higher brain metabolism of the adult cannot be maintained adequately for a period longer than one to 3 minutes by the slow release of anaerobic energy. To determine whether the newborn could avail itself of this anaerobic mechanism when under the effects of morphine it was necessary to learn whether or not morphine shortens the survival period in anaerobiosis. Since it was found (table 2) that newborn rats, injected with morphine and deprived of oxygen, continued to live for nearly as long as their uninjected controls, it was concluded that morphine permits the anaerobic development of energy.

Although there is probably no difference in the availability of anaerobic energy immediately before and after birth, a difference does exist in the availability of oxygen to the newborn and the fetus. The newborn is exposed to an atmosphere of oxygen, the fetus is not. The ability of the respiratory centers of the newborn to obtain sufficient energy anaerobically permits the newborn to respire oxygen and thus to make available energy released by aerobic processes. The fetus *in utero* possesses the same anaerobic source of energy as the newborn but the functions maintained by the anaerobic energy do not procure additional atmospheric oxygen for the organism. In addition, the respiration is depressed and so the normal supply of oxygen to the fetus

fetus *in utero* is comparable to a part of the mother's body in its dependence upon her blood for oxygen and is deprived of aerobic energy with depression of maternal respiration.

LATE EFFECTS OF MORPHINE. Despite the great resistance of the newborn rats to the acute lethal effects of morphine it has been observed that they die of the late effects of morphine with doses far below those lethal to adults. The newborn succumb as a result of prolonged anoxia. This prolonged action of morphine in the newborn may be attributed to a slower rate of detoxification if it can be shown that this process proceeds more slowly in the newborn. The late death of adults, on the other hand, may be a result of intercurrent infections, since the adult had previously recovered from the depressant effects of the morphine. Such intercurrent infections have been observed by Schlossmann (2). Although many adults had convulsions there did not seem to be a direct relationship between these and the lethal effects. None of the newborns injected with morphine exhibited convulsions.

#### SUMMARY

Lethal doses of morphine sulphate were administered to 116 adult rats, 300 newborn rats, and 11 pregnant rats at full term. The newborn rats were more resistant to acute respiratory failure than the adult rats, but the fetuses were about equal in susceptibility to their mothers. Probable causes for the characteristic susceptibility of fetal, newborn, and adult rats to lethal doses of morphine are discussed.

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# THE THERAPEUTIC EFFECTS OF REPEATED SUBCURATIVE DOSES OF MAPHARSEN ON *T. EQUIPERDUM* INFECTIONS IN THE RAT

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Since the introduction of the arsenicals many different opinions have been held in regard to the optimum quantity of arsenical to administer at any one time and the interval between treatments necessary for the clinical control of syphilis. Wechselman (20) in 1911 reported almost miraculous results with a single injection of arsphenamine. Ehrlich (4) suggested giving one injection intravenously, followed in 48 hours with an intramuscular injection which was to exterminate any remaining spirochetes by its prolonged action. Wechselman (21) later gave repeated injections of both arsphenamine and neoarsphenamine. Pollitzer (11) in 1926 recommended 0.1 gram of arsphenamine for each 25 pounds of body weight every day for three days.

The present most widely accepted clinical routine for the treatment of syphilis is the method employed by the "Cooperative Clinical Group" (14), by which method an arsenical drug, arsphenamine, neoarsphenamine or mapharsen, is administered in courses of 12 to 20 injections at weekly intervals. Treatment is intensive for at least 18 months and is supplemented with bismuth or mercury. Paget (10), in a recent survey of the long term (5-10 year) efficiency of presumptive cures by this method, reported an incidence of permanent cures of 90% under prolonged continuous treatment, falling to 68% with 6 months of continuous treatment and to 47% with 6 months of intermittent treatment.

The selection of the time interval between injections, however, is purely arbitrary and has resulted largely from the fear of producing cumulative arsenical poisoning. It by no means follows that the administration of any arsenical at intervals of 3 to 7 days represents the maximum therapeutic effect that could be derived from that particular arsenical drug. A number of clinical attempts have been made to investigate the possibilities of shorter time-intervals between injections. In 1935 Chargin, Leifer, and Hyman (1) reported their results on the administration of neoarsphenamine by means of the intravenous drip technique in the treatment of early syphilis. With this method the patient is hospitalized and 12 doses of the arsenical are administered at intervals of 1 hour. The procedure is repeated daily for 5 days. The results were so promising that a further study was made by Hyman (7, 8) and his associates in 1939. In this series of cases they reported the production of 85% of clinical cures.

Although all clinical treatment of syphilis depends upon the repeated adminis-

<sup>1</sup> Presented as a thesis in partial fulfilment of the requirements for the degree Master of Science at the University of Minnesota.

tration of subcurative doses of the therapeutic agent, almost all studies of the curative effects of antisyphilitic drugs in experimental animals have been made on the basis of the injection of a single completely curative dose. In the comparatively few studies of the effects of subcurative doses Voegtlin (17) found that "in order to kill all of the parasites, a well-defined minimum concentration must be established below which only part of the parasites are killed." Probey (13) and Voegtlin and Dyer (19) found that the sterilizing action of arsphenamine depended upon the amount of arsenic injected. Kolmer (9), employing a sample of arsphenamine having a single minimum curative dose of 4 mgm./kgm., obtained equivalent cures from 3 daily injections of 3 mgm./kgm.; 6 daily injections of 2 mgm./kgm.; 5 semi-weekly injections of 2.6 mgm./kgm.; or 6 weekly injections of 2.6 mgm./kgm. With neoarsphenamine having a single M. C. D. of 6 mgm./kgm. he obtained equivalent cures with 3 daily injections of 5 mgm./kgm.; 6 daily injections of 4.5 mgm./kgm.; or 4 semi-weekly injections of 5 mgm./kgm.

The lack of adequate information in regard to the therapeutic effects of subcurative doses, and the current interest in the development of more rapid clinical cures in the treatment of syphilis suggested an investigation of the curative effects of the serial administration of various subcurative doses of mapharsen in the treatment of *T. equiperdum* infections in rats. Since this study was completed, Eagle and Hogan (4) have presented a preliminary report of a study of the effects of serial dosages of mapharsen in the treatment of syphilis in rabbits, but have presented no experimental details.

**MATERIALS AND METHODS.** Albino rats of the Wistar strain, of 125-150 gm. weight, bred in our own colony, were employed throughout the investigation.

The organism employed was *Trypanosoma equiperdum*.<sup>2</sup> The organism was maintained in rats and guinea pigs. When it was obtained from guinea pigs it was stabilized by several passages through rats before being used.

Rats were inoculated by the intraperitoneal injection of 1 cc. of a suspension of blood in 0.65% sodium chloride, containing 1 organism per field under the 4 mm. objective.

Treatment was commenced 24 hours after inoculation with the organism. A control inoculated rat was included in every series. The 25 control rats employed died in an average time of  $4.6 \pm 0.8$  days ( $110 \pm 20$  hours) after inoculation. The range was from 3 to 8 days.

The mapharsen was administered by the intramuscular route because of the necessity for repeated injections. Gruhzit (6) and Voegtlin (18) have shown that mapharsen has approximately the same therapeutic efficiency by either intravenous or intramuscular injection. Injections were made in alternate legs to avoid the formation of abscesses or dry slough.

The curative efficiency of the sample of mapharsen employed was first determined. Groups of inoculated rats were then given serial injections of various fractions of the M.C.D. at a series of time intervals. The time intervals employed were 6, 12, 24, 48, 72, and 96 hours. Longer time intervals between injections were not investigated because untreated control animals died in an average time of 110 hours after inoculation with the organism.

Ten rats were employed in each group at each dosage level and time interval. In the

<sup>2</sup> The strain of *T. equiperdum* employed in this study was kindly furnished to us through the courtesy of Dr. A. L. Tatum, Professor of Pharmacology, University of Wisconsin.

majority of groups, series of injections from 2 to 20 by increments of 2 injections were employed, with one rat at each injection level. In some groups 2 rats at each injection level were employed from 2 to 10 injections. A total of 230 rats was employed in the investigation.

All animals surviving the projected course of treatment were kept under observation for 30 days after completion of treatment unless death took place before that time. Animals surviving 30 days after completion of treatment, examination of the tail blood being negative for trypanosomes, were considered cured.

**RESULTS.** *Single dose therapeutic efficiency of mapharsen.* The curative activity of mapharsen when administered as a single dose to rats infected with *T. equiperdum* was determined by injecting the drug into two series of rats at dosages through the range of 0.6 to 2.0 mgm./kgm. by increments of 0.2

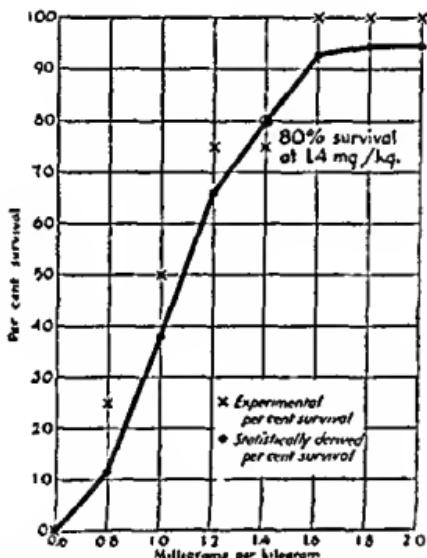


FIG. 1. PER CENT SURVIVAL OF RATS INOCULATED WITH *T. EQUIPERDUM* FOLLOWING THE INJECTION OF A SINGLE INTRAMUSCULAR DOSE OF MAPHARSEN. M.C.D. (50%) AT 1.4 MG.M./KGM.

mgm./kgm. The results, integrated for both deaths and survivals after the manner of Dragstedt and Lang (2), previously employed by Wright (22) in digitalis assays, are shown in fig. 1. For the purposes of this study the minimum curative dose (M. C. D.) was taken as the dosage at which 80% of animals injected with that dosage may be expected to survive, and this was found to be 1.4 mgm./kgm. This is an excellent agreement with the results of Tatum and Cooper (15) who found a dosage of 1.2 mgm. per kilogram gave the same percentage survival when injected intravenously.

The curative effects of repeated subcurative dosages of mapharsen were then determined by the injection of progressively smaller percentages of this M. C. D. into succeeding groups of animals.

*Injections of 1.0 mgm. per kilogram.* This dosage represents approximately

70% of the single minimum curative dose. The percentage survival graph (fig. 1) shows that approximately 38% of animals might be expected to be cured by a single dose of this magnitude.

Serial injections of this dosage were made at the 96 hour time interval for series of 2, 4, 6, and 8 injections employing two rats in each group. All animals survived this treatment. (Fig. 2.)

The curative efficiency of the repeated administration of a dosage representing 70% of the single dose M.C.D. of mapharsen appears to be such that two or more injections are uniformly curative even when spaced 96 hours apart, a time interval which closely approached the mean survival time for untreated control animals.

*Injections of 0.8 mgm. per kilogram.* This dosage represents approximately 55% of the single M.C.D. Only 11% of animals may be expected to survive a single injection of this dosage (fig. 1).

Serial injections were made at time intervals of 6, 12, 24, 48, 72, and 96 hours, employing two animals at each injection level for 2, 4, 6, and 8 injections at the four shorter time intervals, and a single animal at each injection level from 2 to 20 injections at 72 and 96 hours. All animals survived at all dosage levels for all time intervals (fig. 2).

The curative efficiency of a dosage of 55% M.C.D. of mapharsen, therefore, also appears to be such that two or more injections (aggregating a minimum of 1.1 M.C.D.) are uniformly curative even when spaced 96 hours apart.

*Injections of 0.6 mgm. per kilogram.* Although the dosage employed here represents only approximately 40% of a single M.C.D., and no animals may be expected to survive when treated with only a single injection (fig. 1), nevertheless this dosage still appears to possess considerable curative efficiency provided the serial dosages are administered at 6 or 12 hour time intervals (fig. 2). At the 6 hour time interval 2 of the 9 animals died after receiving injections of 2 and 6 doses respectively, both dying 15 days after cessation of treatment. At the 12 hour time interval 2 of the 10 animals died after receiving 4 and 14 injections. For these two time intervals the curative effect of mapharsen appears to be such that four or more injections (aggregating a minimum of 1.6 M.C.D.) will effect a cure in approximately 85% of animals, although no animals will be cured by the administration of a single injection.

At the longer time intervals this dosage is definitely less efficient, although little essential difference is seen between the results at the 24, 48, and 72 hour time intervals,—approximately one-half the animals survived in each case. The animals that died received an average of 8 injections, those that survived an average of 12.5 injections. At the 96 hour time interval, the time between injections permits the infection to overpower the treatment and all animals died after receiving an average of 3.4 injections.

*Injections of 0.4 mgm. per kilogram.* This dosage represents approximately 30% of a single M.C.D. No animals would be expected to be cured by the administration of only a single injection (fig. 1).

This dosage appears to be at a level from which comparatively little in the

### Fraction of Minimum Curative Dose Injected

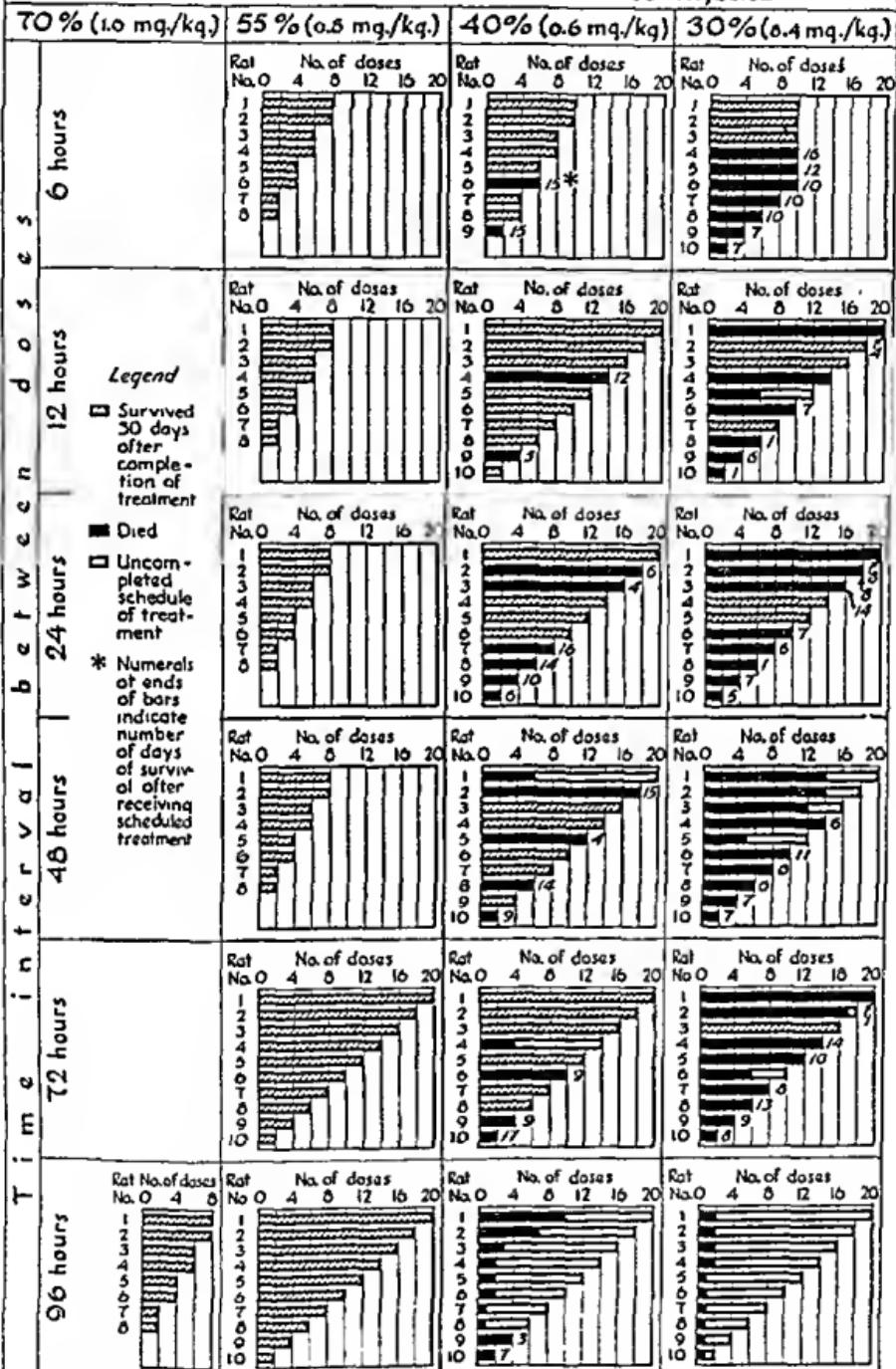


FIG. 2. CURATIVE EFFECTS FROM SERIAL INTRAMUSCULAR INJECTIONS OF VARIOUS FRACTIONS OF THE M.C.D. (80%) OF MAPHARSEN IN RATS INOCULATED WITH T EQUIPERDUM

way of therapeutic effects is to be expected. Even at a time interval of 6 hours, which, in spite of the small individual dose resulted in the administration of more than a single M.C.D. in the course of 24 hours, no animals survived if given less than 10 injections and only 50% of the 6 rats receiving 10 injections survived. At the 12 hour time interval 3 of the 10 animals survived after receiving an average of 14 injections, while the remaining 7 died after receiving an average of 9 injections.

Again there is no marked difference between the results obtained at 24, 48 and 72 hours, 90% of the animals dying after having received an average of 10 injections, the remaining 10 per cent surviving after receiving an average of 14 injections. At the 96 hour time interval no animal survived beyond the second dose.

**DISCUSSION.** The administration of repeated subcurative doses of organic arsenical drugs in the experimental evaluation of these compounds rather than the administration of single completely curative doses more nearly parallels the clinical treatment of syphilis, yet has been very inadequately studied. The results obtained in this investigation may be considered conveniently from two standpoints, (1) the effect of dosage and (2) the effect of time interval between injections.

From the standpoint of the effect of dosage, there appears to be a comparatively sharp break in the efficiency of dosages smaller than 50% of a M.C.D. Dosages in excess of this were uniformly curative in two or more doses at all time intervals, even at intervals of 96 hours. This apparently takes place because the initial dose destroys a sufficiently large number of the organisms that they do not restore the infection to its former level by the time of the next injection, hence two or more injections are uniformly curative.

At dosages below 50% M.C.D. the results are much more dependent upon the time interval between injections, 85% survival (the equivalent of one M.C.D.) being obtained at the 6 and 12 hour intervals with 40% M.C.D. while at 96 hours no animals survived treatment with the same dosage. With dosages of 30% M.C.D. the curative efficiency of mapharsen becomes very small even at the 6 and 12 hour time intervals, no animals surviving if given less than 8 injections and not more than 50% of the animals surviving after receiving 10 or more injections. At time intervals in excess of 12 hours the curative efficiency of 30% M.C.D. is practically nil.

From the standpoint of the effect of time interval, a number of points emerge. Provided the initial dose is greater than 50% M.C.D., the time interval between doses appears to have little effect, the effect of a single dose (although much less than one M.C.D.) being such that most of the organisms are destroyed by this dosage and are unable to multiply sufficiently rapidly to restore the infection to its former level by the time of the next dose.

With dosages of less than 50% M.C.D., however, the time factor plays an important role. With dosages of both 40% and 30% M.C.D. the results obtained at the 6 and 12 hour time intervals are appreciably better than at the longer time intervals. These results indicate that the duration of the curative

efficiency of a single injection of mapharsen in the rat does not greatly exceed 12 hours. This fact is amply confirmed by the results of other investigations by Peters and Wright (11) to be published shortly. For the longer time intervals there is little difference in the results obtained at injection intervals between 24 and 72 hours. Injections of the drug at 96 hour intervals, however, appear to allow such a long time to elapse between the cessation of the curative effect and the next injection that the animals succumb to the infection after 2 to 4 injections.

These results indicate that the duration of the trypanocidal action of mapharsen is sufficiently short that maximum clinical benefit cannot be expected to be obtained from the injection of the drug at weekly or even semi-weekly time intervals. Maximal therapeutic results from the clinical use of mapharsen appear to be indicated by patterning the administration of the drug after the method that has been adopted so successfully in the treatment of systemic bacterial infections with compounds of the sulfonamide series, namely, the administration of repeated subcurative doses of the drug at a time interval sufficiently short that a constantly spirocheticidal concentration of the drug will be maintained in the blood stream night and day for a period of time sufficiently long to produce a high percentage of "permanent cures" in a single course of treatment. The time interval elapsing between injections should apparently not exceed 12 hours.

The dosage should be the maximum that the incidence of clinical toxicity will permit. The upper limit of this has probably been indicated by Hyman and coworkers (1, 3, 7, 8) who gave 0.24 gm. of mapharsen daily over a period of 10-12 hours by the intravenous drip technic and by Thomas and Wexler (16) who gave 0.12 gm. daily in two doses. With these dosages both groups of workers experienced a greater frequency of toxic reactions than is obtained with the more conservative weekly or semi-weekly treatment.

The low curative value of our dosages representing 30% M.C.D., on the other hand, confirms Voegtlin's finding of the necessity for "a well defined minimum concentration," and indicates that the injection of dosages that are too small, even though injected at frequent intervals, may be comparatively wasteful therapeutically from failure to achieve the necessary minimal curative blood level of the arsenical compound, yet potentially dangerous from the standpoint of the production of toxic manifestations.

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